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## Circadian rhythms and cancer

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### Abstract

The circadian clock is an endogenous time keeping system shared by most organisms. In mammals, a master pacemaker in the hypothalamus orchestrates temporal alignment of behavior and physiology by transmitting daily signals to multiple clocks in peripheral tissues. Disruption of this communication has a profound affect on human health and has been linked to diverse pathogenic conditions, including cancer. At the center of the molecular circadian machinery is a set of clock genes, generating rhythmic oscillations on a cellular level. In the past several years, research from different fields has revealed the complexity and ubiquitous nature of circadian regulation, uncovering intriguing associations between clock components and cellular pathways implicated in tumorigenesis. In this review, we discuss the emerging role of circadian genes in hematological and hormone-related malignances. These new insights suggest that manipulating circadian biology as a way to fight cancer, as well as, other life threatening diseases is within the realm of possibility.

### Keywords

circadian; hematopoietic system; breast cancer; prostate cancer; tumorigenesis

### Introduction

For most organisms, the ability to adapt to their environment enhances overall well-being and provides a selective advantage. Indeed, organisms ranging from cyanobacteria (among the earliest cellular life forms) to mammals developed an internal time keeping system—termed circadian—allowing them to anticipate one of the most profound environmental signals, the daily cycle of light/dark (translated from Latin, circa diem: about a day). For ancient life forms, restricting the UV-sensitive S-phase of the cell cycle to nighttime, thereby avoiding strong irradiation-induced DNA damage, may have had a selective value, what is known as the “escape from UV” hypothesis.<sup>1</sup> From this humble beginning, circadian influence in modern organisms has found its way to an unparalleled array of biological activities synchronizing cells, organs and organisms with earth’s daily rotation around its axis. In fact, the ability to keep time is a basic characteristic of life on plant earth.

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The first report of endogenous rhythms is attributed to the French astronomer de Mairan who, in 1729 demonstrated that daily leaf movements of a plant persists in constant darkness, and remarkably went on, to propose that this phenomenon is related to sleep patterns of bedridden patients.<sup>2</sup> Major advances in elucidating the molecular intricacies underlying circadian rhythms began in the early 70s with the discovery of clock genes in *Drosophila*. However, it was the decade of the 90s that saw an explosion of data in the field, leading to the current model of the clock mechanisms.<sup>3</sup> Research over the last ten years added to these discovers and revealed the complexity and extensiveness of circadian regulation not only within the clock machinery but also as it relates to other molecular networks. The challenge in the coming decade is to convert these molecular insights to benefit life in the real world.

Although the clock components are not conserved between kingdoms, the molecular mechanisms underlying circadian rhythms are universal in all model systems. In mammals this system consists of a central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus, input pathways transmitting temporal information (primarily light) to the pacemaker, and output pathways conveying information from the central pacemaker to organs throughout the body.<sup>4-8</sup> At the core of the molecular circadian machinery are interlocked transcriptional/translational feedback loops generated by a set of clock genes.<sup>9</sup> The heterodimeric transcription factor complex, Clock/ Bmal1 binds to E-boxes in the promoter of Period (Per) and Cryptochrome (Cry) genes. Per and Cry proteins heterodimers and upon reaching a critical concentration, attenuate Clock/ Bmal1 activity, thereby forming the major negative feedback loop. A secondary feedback loop is composed of two subfamilies of nuclear hormone receptors, Rev-erb and Ror, which directly regulate the core feedback loop, increasing its robustness. Further levels of regulation are achieved by posttranslational modification and chromatin remodeling that are crucial for maintaining circadian rhythmicity.<sup>10-12</sup>

The same clock genes which make up the SCN oscillator are expressed, and, moreover, exhibit temporal oscillations in most peripheral tissues.<sup>4,5</sup> Circadian oscillations can be detected even in established cell lines.<sup>13,14</sup> The peripheral clocks are synchronized by the central clock and also have inherent circadian properties. Remarkably, the clock transcriptional machinery is not restricted to generate temporal expression of clock genes, but extends to regulate hundreds of non-clock genes in a tissue specific manner. Expression profiling studies demonstrated that in any given tissue, up to 15% of transcripts are clock controlled genes.<sup>15,16</sup> The Clock/Bmal1 complex directly controls the expression of some of these genes, whereas others are indirectly regulated through circadian expression of relevant transcription factors. Subsequently, these rhythmically expressed genes integrate circadian information into multiple organismal and cellular processes such as hormone secretion, aging, metabolic pathways, DNA damage response and cell cycle progression. The importance of this multifaceted system to human physiology implies that disruption of circadian function will inevitably lead to disease.<sup>8,17</sup> Indeed, circadian deregulation has been linked to an impressive array of pathologic conditions including diabetes, cardiovascular disease, depression and cancer. Herein, we review some of the recent findings linking the circadian system to cancer and focus primarily on hematopoietic and hormone-related malignances.

## Hematopoietic System

Circadian rhythmicity in hematopoietic cell numbers was reported as early as the 50s. Later studies revealed expression of clock genes in multiple hematopoietic cell types.<sup>18-23</sup> Furthermore, circadian genes appear to be temporally expressed and developmentally regulated in various murine and human hematopoietic lineages including stem/progenitor cells. Additionally, circulating levels of hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), and interleukins-2, -6 and -10 also display daily oscillations. At the cellular level, and similar to other tissues, the circadian apparatus and clock specific proteins regulate cell cycle and apoptotic pathways in hematopoietic cells.<sup>24</sup> Hematopoiesis also does not escape circadian regulation at the systematic level (as discussed in more details below).<sup>25</sup>

Circadian regulation is critical to normal hematopoiesis; *pari passu*, disruption of circadian function has been implicated in hematopoietic neoplasms. The significance of proper circadian regulation to cell proliferation and neoplastic transformation was illustrated by studies in mice containing core clock gene mutations (Table 1). When kept in constant darkness *Per2* mutant mice become arrhythmic; remarkably, *Per2* deficient mice are also prone to develop cancer.<sup>26</sup> In response to radiation these mice have a 10-fold increased incidence of lymphomas relative to wild-type controls. Temporal expression of genes involved in cell cycle regulation and tumor suppression, such as *Cyclin D1* and *A*, *c-Myc*, *Mdm2* and *Gadd45 $\alpha$* , is deregulated in *Per2* mutant mice. In particular, *c-Myc* is under direct circadian control through a clock-responsive E-box in its promoter. In additional studies, after partial hepatectomy, livers from *Cry* deficient mice were found to regenerate slower than those from normal mice. In this model, the circadian clock controls the  $G_2/M$  transition by directly regulating *Wee1* kinase, an inhibitor of the *cdc2/cyclin B1* complex, the major cyclin complex governing the  $G_2/M$  transition.<sup>27</sup>

Analyzing primary patient samples shows that *Per* gene expression is downregulated in various types of leukemias and lymphomas (Table 2). For example, *Per* gene expression is reduced in peripheral blood of chronic myeloid leukemia (CML) patients compared with healthy individuals.<sup>28</sup> Furthermore, CpG sites of *Per2* and *Per3* promoters are often methylated in CML patients, with a significant increase frequency in *Per3* methylation in patients during blast crisis compared with those in the chronic phase of the disease. We analyzed *Per2* expression in a large number of leukemia<sup>29</sup> and lymphoma (Thoennissen and Koeffler HP, unpublished) patient samples, as well as control normal mononuclear bone marrow and tonsils. *Per2* levels were markedly down-regulated in acute myeloid leukemia (AML) and diffuse large B cell lymphoma (DLBCL). Genetic variants of the clock genes are associated with a risk for non-Hodgkin's lymphoma (NHL),<sup>30,31</sup> and epidemiological studies noted that men who work at night have an increased risk for NHL.<sup>32</sup> *In vitro* cell culture experiments showed that forced expression of *Per2* in human and murine AML and pro-B lymphoid cell lines led to growth inhibition, cell cycle arrest, apoptosis and loss of clonogenic ability.<sup>29</sup>

Tissue specific transcription factors acting as oncogenes or tumor suppressors may also interfere with proper clock function. CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors critical for proliferation and differentiation in multiple cell types including hematopoietic cells. Deregulation of C/EBPs plays a role in a number of hematologic malignancies, with C/EBP $\alpha$  being a bona fide tumor suppressor in leukemia.<sup>33</sup> Using gene expression profiles, we found that the core clock genes Per2 and Rev-Erba, as well as Dbp (albumin site d-binding protein, a circadian output gene), are C/EBP targets.<sup>29</sup> In vitro studies in human leukemic cell lines suggest that induction of Per2 by C/EBP $\alpha$  contributes to, at least some of the tumor suppressive properties C/EBP $\alpha$ . Interestingly, C/EBPs themselves are rhythmically expressed in various tissues and have been proposed to act as circadian transcriptional regulators,<sup>34</sup> that may form an additional circadian feedback loop (Fig. 1). C/EBP family members are also key regulators of G-CSF, the most important cytokine regulating neutrophil production. A recent study showed that C/EBPs regulate G-CSF-induced myeloid differentiation via a feedback loop involving the NAD<sup>+</sup>-Sirt1 metabolic pathway,<sup>35</sup> which is under direct circadian control<sup>36,37</sup> (as discussed below). These new discoveries together with bioinformatics and genome-wide screening approaches,<sup>15,16,38-44</sup> reveal an ever expanding network connecting the circadian clock genes to a multitude of cellular pathways.

The ubiquitous nature of cellular and systemic circadian regulation suggests that understanding circadian intricacies may offer new therapeutic avenues. Recently, chronotherapy, i.e., administration of treatment in coordination with circadian rhythms, has been attracting attention, particularly in the field of oncology.<sup>45,46</sup> Conventional cancer therapeutics usually target proliferating cells and these drugs are often limited by their toxicity to normal tissues. Cancer chronotherapy is based on asynchronies in cell proliferation and drug metabolic rhythms between normal and tumor cells, with the aim of minimizing the damage to normal tissues and maximizing drug efficacy in malignant cells. This approach has been used successfully in several animal models, as well as in patients with advanced stage cancers.

Under physiological conditions, hematopoietic stem cells (HSC) continuously move between the bone marrow and the bloodstream. Artificially induced mobilization of HSC (by cytotoxic drugs or cytokines) from the bone marrow into the peripheral blood along with engraftment of intravenously injected HSC into the bone marrow, are the basis for HSC transplantations. Murine models show that unforced, physiological release of HSC is influenced by circadian fluctuations in expression levels of CXCL12, a chemokine essential for HSC mobilization and its receptor, CXCR4.<sup>25</sup> Neural signals transmitted from the SCN to stromal cells in the bone marrow regulate circadian CXCL12 expression, while rhythmic expression of CXCR4 on HSC is controlled by core clock genes. Furthermore, by controlling the expression of the CXCL12/CXCR4 in the bone marrow niche, circadian rhythms continue to affect HSC mobilization even in the presence of pharmacological agents like the CXCR4 antagonist, AMD3100 or G-CSF, the most common mobilizer used in the clinic.<sup>47</sup> Additionally, circadian variations of bone marrow engraftability were described in a congenic murine transplant model.<sup>48</sup> These findings suggest that simple adjustments in timing of stem cell harvest and infusion may result in higher yields and greater engraftment, respectively. The bone marrow microenvironment is required for growth and survival of not

only normal HSC but also that of leukemic stem cells (LSC). Furthermore, the hematopoietic niche may offer protection to LSC during chemotherapy and by doing so contribute to chemoresistance and disease relapse. Disrupting CXCL12/CXCR4 signaling has been shown to overcome resistance to chemotherapy and to kinase inhibitors in AML.<sup>49,50</sup> In future studies, it will be interesting to determine if chronotherapy can be applied to maximize efficacy of drugs disrupting LSC/niche interactions, with minimum affect on HSC.

Local circadian control in hematopoietic cells may also modulate circadian susceptibility to chemotherapy. A recent study found that mice with different core clock gene mutations show variations in sensitivity to the chemotherapeutic agent cyclophosphamide.<sup>51</sup> Interestingly, the differences were not attributed to changes in drug metabolic pathways but to changes in hematopoietic cells recovery rate, which was dependent on the functional status of Clock/Bmal1. Although neutrophil counts varied only slightly, the survival/recovery of B cells in response to cyclophosphamide showed significant differences between wild-type and core clock gene mutant mice. These findings demonstrate a mechanistic link between core clock factors expressed by immune cells and the sensitivity to conventional cytotoxic treatments.

## Hormone-Related Breast and Prostate Cancers

The secretion of many endocrine and metabolic hormones is controlled by the day/night cycle, and reciprocally circulating levels of hormones influence circadian rhythms. While this bidirectional flow of information is critical for normal physiology, a growing number of studies suggest that circadian variation is particularly relevant to endocrine malignancies. A remarkable example is the finding that overnight shift workers have an increase in hormone-related breast and prostate cancers, as well as other types of malignancies (Table 3).<sup>52-58</sup>

Among the cancers known to be impacted by the circadian clock, breast cancer has received the most attention. In addition to epidemiological studies showing a clear correlation between disruption of circadian rhythms and breast cancer risk, a number of studies reported that Per1 and Per2 expression in both sporadic and familial primary breast tumors is significantly decreased compared to normal breast tissues.<sup>59-61</sup> Expression of Per genes also correlates with expression of other genes implicated in breast cancers including Her2, BRCA1 and estrogen receptor. The deregulation of Per is most frequently associated with methylation of regions of the Per gene promoters. However, a large screen of human breast cancer genomes identified mutations in both Per1 and Per2 genes,<sup>62</sup> supporting their role as tumor suppressors.

Compared with the relative large number of studies addressing circadian function in breast cells, fewer studies have explored the existence of circadian oscillations in the prostate, and molecular understanding of a possible interplay between circadian regulation and prostate cancer is largely unknown. We and others demonstrated circadian expression of the core clock genes in prostate and other male reproductive tissues in mice entrained to 12 hours light/dark cycles.<sup>63,64</sup> On the other hand, rhythmic expression of core clock genes is severely impaired in serum-synchronized human prostate cancer cell lines. Additionally, meta-in-

silico analysis showed that Per1 and Per2 expression is significantly lower in prostate carcinoma patients compared with normal controls, and that Per1/2 levels decreased with progression to metastatic disease. Population-based studies found associations between genetic variants in clock genes and prostate/breast cancer risk and survival.<sup>65-67</sup>

Forced overexpression of either Per1 or Per2 in breast and prostate cancer cells inhibits their proliferation in culture, while silencing of Per1/2 expression by siRNA accelerates their growth.<sup>68,69</sup> Although the molecular mechanisms linking Per genes to breast and prostate cancers are still elusive and likely involve multiple cellular pathways, we recently showed that Per genes link the circadian system to estrogen and androgen signaling, providing at least one possible explanation for the strong association between hormonal related cancers and the circadian clock. The gonadal steroid hormones estrogen and androgen are essential to normal physiology and are important factors in breast and prostate tumorigenesis, respectively. These hormones mediate their effects mainly through the estrogen and androgen receptors (ER and AR) which are members of the nuclear receptor (NR) superfamily. Recent observations demonstrated intimate relationships between NR and central as well as peripheral circadian clocks; this is most notable in metabolic physiology.<sup>70,71</sup> While several steroid receptors and their hormones have been linked to both circadian and reproductive rhythms, the significance of these connections to cancer is largely unknown. We found that Per2 interacts with ER $\alpha$ , and suppresses estrogen-mediated transcription of ER target genes in breast cells.<sup>69</sup> In turn, Per2 itself is an estrogen-inducible gene, suggesting a feedback mechanism that couples the circadian clock to the estrogen pathway. Likewise, we showed that Per1 physically binds to AR.<sup>64</sup> Furthermore, forced expression of Per1 in prostate cancer cells inhibits AR transcriptional activity, including DHT stimulation of PSA. Conversely, silencing of Per1 expression using siRNA is associated with increased transcriptional activity of AR. In turn, activated AR increases the transcription of Per1 associated with the recruitment of AR to an androgen response element in the Per1 promoter. Our results suggest that Per1 acts as an AR co-repressor coupling circadian rhythms to AR signaling in prostate cells (Fig. 2). The finding that Per1/2 are estrogen- and androgen-responsive genes provides a direct link between core circadian components and the ER/AR pathways. Temporal expression of all 49 murine NR was surveyed in murine liver, skeletal muscle and adipose tissues.<sup>70</sup> Although AR was expressed in these metabolic tissues, its level did not show circadian expression. Because circadian regulation of gene expression is tissue-specific, we examined whether AR is rhythmically expressed in the prostate. Remarkably, AR mRNA levels oscillate in prostate tissue of mice entrained to 12-hours light/dark cycles, suggesting novel roles of AR in coupling the peripheral clocks to hormonal regulation.<sup>64</sup> More generally, these studies point to an important role for the circadian regulation in proper prostate function, both locally and in the hypothalamic-pituitary-prostate axis.

Recently, acetylation and other chromatin modifications were discovered to be an intrinsic part of circadian function. The Clock protein acts as a histone acetyltransferase (HAT) that acetylates histones, as well as nonhistone proteins; and Sirt1, best known for its potential anti-aging properties, is a histone deacetylase (HDAC) that counteracts Clock.<sup>72,73</sup> Additionally, activation of Hdac3 by the NR co-repressor, NcoR1, is a key step coupling epigenetic regulation to circadian and metabolic physiology.<sup>74</sup> AR transcriptional activity is



regulated by posttranslational modifications including acetylation and both Sirt1 and Ncor1 function as AR co-repressors.<sup>75-77</sup> Sirt1 also regulates prostate cancer cell growth and chemoresistance in androgen-refractory cells.<sup>78</sup> A potentially fruitful area of future studies is to examine whether circadian mediated modifications of acetylation plays a role in AR-dependent and -independent prostate cancer cell growth and drug sensitivity.

Any discussion of circadian hormone-related cancer interplay cannot be completed without the mention of melatonin, the main hormone relaying the circadian rhythm to the peripheral organs. In addition to its well-known role in circadian physiology, recent studies revealed that melatonin also has anti-tumor activities in various human cancers, in particular breast, colon, lung, melanoma and leukemia.<sup>79</sup> A variety of mechanisms have been suggested to explain how reduced melatonin production (occurring artificially upon exposure to light at night, and physiologically with aging) may exaggerate cancer risk. That notwithstanding, melatonin inhibition of breast and prostate cancer cell growth has been attributed at least in part, to its interaction with estrogen and androgen signaling pathways.<sup>80,81</sup> For example, in human breast cancer cells melatonin reduces the expression and activity of aromatase, a key enzyme in estrogen biosynthesis. In human prostate cancer cell lines melatonin induces nuclear exclusion of AR. Small-scale studies showed that combination of melatonin and tamoxifen (used to treat estrogen-positive breast cancer) can have modest synergistic benefits, and that melatonin may enhance the effects of some chemotherapeutic drugs used to treat breast and prostate cancer patients. ER and AR are already targets of widely used drugs; understanding their circadian regulation may therefore have tangible clinical implications.

In summary, over the last decade a wealth of data from interdisciplinary fields revealed that circadian regulation is hardwired into almost every aspect of human physiology. Though much remains unanswered, the notion that pharmacological manipulation of circadian genes can one day be applied to help prevent and treat complex multistep diseases like cancer, may be more than just a dream away.

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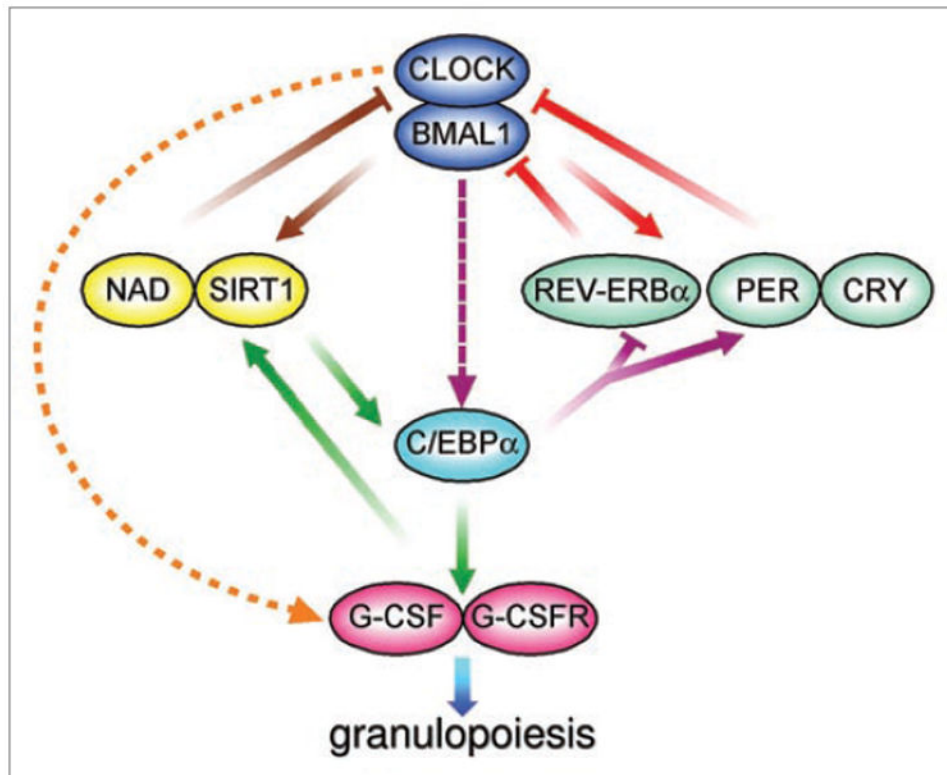
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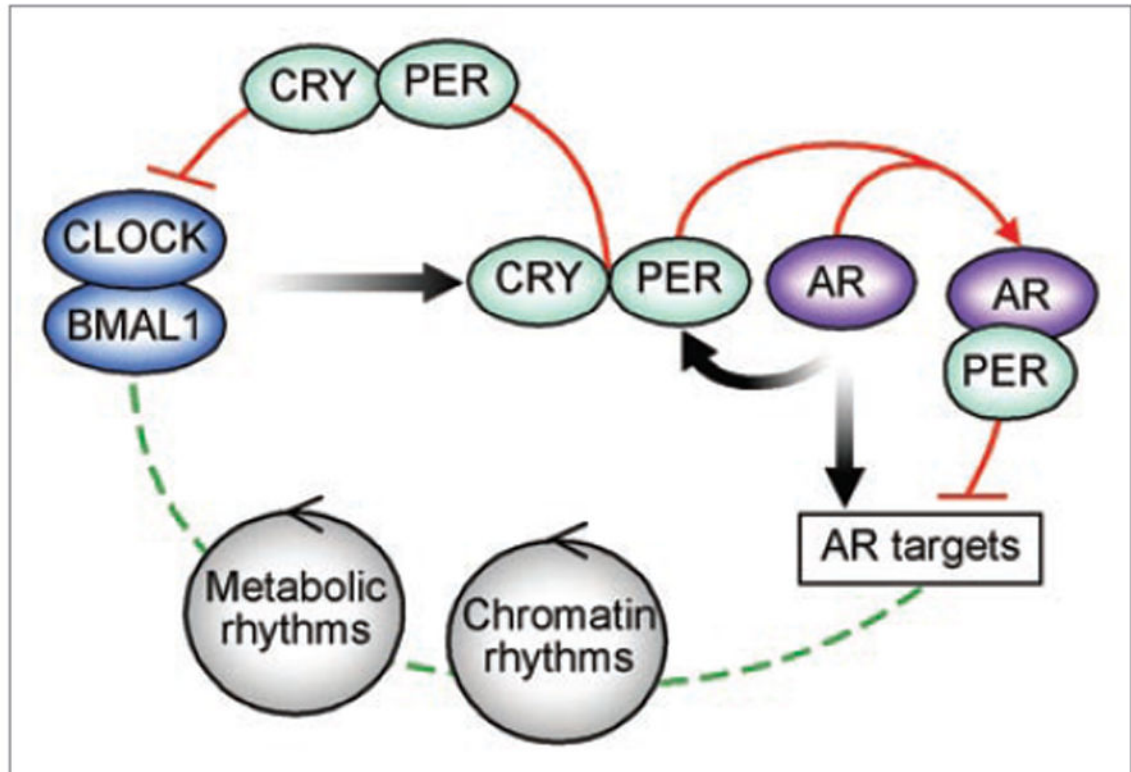
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**Figure 1.**

Connections between circadian cycles, C/EBPs and the NAD<sup>+</sup>-Sirt1 pathway may affect G-CSF induced granulopoiesis. The circadian oscillator is composed of transcriptional feedback loops (Red). Clock and Bmal1 drive expression of core clock genes Per, Cry and Rev-Erba which in turn, inhibit Clock/Bmal1 activity. Throughout the body, these clock cycles are embedded into tissue-specific pathways to sustain local rhythmic physiologies. The Clock/NAD<sup>+</sup>-Sirt1 feedback loop (brown) couples circadian oscillations to metabolism. Diurnal cytokine patterns link circadian regulation to hematopoietic processes (orange). C/EBP transcription factors may form a feedback loop with the circadian clock (purple) as they regulate core clock genes and are themselves rhythmically expressed in some tissues. In myeloid cells, C/EBPs are part of a feedback loop (green) that regulates granulocyte colony stimulating factor (G-CSF) and G-CSF receptor (G-CSFR) in a NAD<sup>+</sup>-Sirt1 dependent manner, leading to induction of granulopoiesis.



**Figure 2.**

Per couples the circadian cycle to the androgen response. Clock and Bmal1 activate the transcription of core clock genes (e.g., Per and Cry) as well as tissue specific rhythmic genes (e.g., AR). Per and Cry heterodimerize and inhibit the Clock/Bmal1 complex. Tissue specific transcription factors like AR, additionally regulate the expression of core clock genes. In turn, Per binds to AR and inhibits AR transcriptional activity. Metabolic and chromatin remodeling cycles further integrate into the circadian control of normal cellular function.



**Table 1**

Disruption of circadian clock genes associated with dysregulated cell proliferation and cancer in murine models

Mouse genotype	Mouse phenotype	Reference
Clock-deficient	High apoptotic and low proliferation rate in lymphocytes and thymocytes; low proliferation rate in embryo-derived fibroblasts; accelerated aging in response to radiation; reduced hematopoietic repopulation after chemotherapy	38, 51, 82
Bmal1 KO	Premature aging associated with increased levels of reactive oxygen	83
Per2-deficient	Increase in tumor development and reduced apoptosis in thymocytes in response to radiation; develop intestinal polyps	26, 84
Apc-mutant/Per2-deficient	Increase in intestinal polyps compared with <i>Apc<sup>Min/+</sup></i> mice	84
Cry1,2 KO	Delayed liver regeneration by controlling G <sub>2</sub> /M transition	27
p53 KO/Cry1/2 KO	Reduced cancer risk compared with p53 KO mice	85

Abbreviations: KO, knockout.

**Table 2**

## Circadian clock genes abnormalities in cancer

Abnormality	Cancer type	Reference
Per gene mutation	breast, colorectal	62
Deregulation of Per gene expression	breast, colorectal, endometrial, lung, CLL, DLBL, AML, CML	28, 29, 59-61, 86-89
Single nucleotide polymorphisms in circadian genes associated with cancer risk and patient survival	breast, prostate, NHL	30, 31, 65-67
Per1 translocation	CML	90

Abbreviations: CLL, Chronic lymphocytic leukemia; DLBCL, Diffuse large B-cell lymphoma; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin's lymphoma.

**Table 3**

Abnormal circadian rhythms associated with cancer in animal models and humans

	<b>Abnormality</b>	<b>Outcome</b>	<b>Cancer type</b>	<b>References</b>
Mice	Disruption of circadian rhythm either by SCN ablation or chronic jet lag	accelerated malignant growth and shortened survival	transplantable osteosarcoma and pancreatic adenocarcinoma	91, 92
	Disruption of circadian rhythms in night-shift workers	independent risk factor for cancer	NHL, breast, colorectal, endometrial, and prostate cancers	32, 52-58
Human	Flattened or abnormal diurnal cortisol rhythms	predictor of survival	metastatic breast cancer	93
	Poor 24-h rest/activity rhythms	independent predictor for overall survival	metastatic colorectal cancer patients receiving chemotherapy	94

Abbreviations: NHL, non-Hodgkin's lymphoma.