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Cancer, hear my battle CRY

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Abstract

Circadian clocks are cell-autonomous self-sustaining oscillators that allow organisms to anticipate environmental changes throughout the solar day and persist in nearly every cell examined. Environmental or genetic disruption of circadian rhythms increases the risk of several types of cancer, but the underlying mechanisms are not well understood. Here, we discuss evidence connecting circadian rhythms—with emphasis on the cryptochrome proteins (CRY1/2)—to cancer through in vivo models, mechanisms involving known tumor suppressors and oncogenes, chemotherapeutic efficacy, and human cancer risk.

Keywords

cell cycle; circadian clocks; circadian rhythm; cryptochromes; DNA repair; oncogenes carcinogenesis

1 | INTRODUCTION

Circadian rhythms synchronize physiological processes and behaviors to zeitgebers (external timing cues) such as light and food availability. The ability to maintain an internal sense of time via a protein-based molecular oscillator is widespread throughout phylogeny. The suprachiasmatic nucleus (SCN) located in the anterior hypothalamus is the master clock that drives rhythms in hormone production, locomotor activity, and feeding behavior, which in turn entrain peripheral clocks located throughout the body.[1] Keeping time throughout the body is important for overall health and well-being.

At the molecular level, mammalian circadian clocks are based on a transcription-translation feedback loop (TTFL) that regulates gene expression and contributes to oscillations of protein abundance. A heterodimer composed of circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1) drives expression of the CLOCK:BMAL1 repressors periods (PER 1–3) and cryptochromes (CRY 1–2) and other clock-controlled genes. An additional feedback loop in which the nuclear hormone receptors (NRs) RORα/γ and REV-ERBα/β drive rhythmic expression of the *Bmal1* transcript is also

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CONFLICT OF INTEREST

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required to sustain rhythms.[2] This negative feedback produces approximately 24-hour oscillations in CLOCK:BMAL1 transcriptional activity, leading to oscillating expression of a significant fraction of the transcriptome and of the proteome in all mammalian organs that have been examined.[3] Indeed, ~50% of mammalian genes are rhythmically expressed.[4,5] These molecular rhythms modulate physiological processes such as metabolism, DNA repair, and inflammation—functions important for cancer growth.[6] Deletion, mutation, or dysregulation of some core clock proteins has been implicated in cancer.[7,8]

The first evidence that environmental disruption of circadian rhythms influences breast cancer incidence was observed in the 1960s.[9] Subsequently, through numerous epidemiological studies, the World Health Organization (WHO) designated circadian rhythm disruption a probable carcinogen.[10] Circadian disruption can occur through a wide variety of human behaviors, most commonly shift work or trans-meridian travel.[11] This disruption can be modeled experimentally in mice by altering their light schedules. In genetically engineered mouse models of breast, lung, and liver cancer,[12–14] as well as xenografts of osteosarcoma and pancreatic adenocarcinoma,[15] exposure to altered lighting conditions that mimic rotating shift work or chronic jet lag increased tumor formation and progression.

Cryptochromes evolved from bacterial light-activated DNA repair enzymes.[16] In insects, the evolution of CRYs' transcriptional repressor function seems to have co-occurred with loss of direct light sensing.[17] Mammalian CRYs lack both innate light sensitivity and catalytic DNA repair activity but are sensitive to metabolic cues[18] and may be regulated by magnetic fields.[19] Their evolutionary relationship to DNA repair enzymes has motivated investigation of their potential role in protecting the genome by other means. In this review article, we discuss evidence connecting cryptochromes and cancer through in vivo mouse models of cancer and mechanistic or correlative studies involving tumor suppressors, oncogenes, and chemotherapy.

2 | DOES MELATONIN PROVIDE PROTECTION AGAINST CANCER?

Because the SCN drives daily oscillations in the production and secretion of hormones, including predominantly glucocorticoids[20] and melatonin,[21] their levels in the circulation are altered by circadian disruption. Melatonin is synthesized by the pineal gland and its production is controlled by both light and circadian rhythms. Circadian regulation of melatonin drives its synthesis and secretion during the dark phase; exposure to light at night directly suppresses melatonin production; and insufficient exposure to daytime light results in delayed and/or reduced melatonin accumulation,[21] which impacts sleep and likely other physiological systems as well. Melatonin modulates glucose homeostasis[22–24] and has been reported to suppress cell and tumor growth.[25] Many tumors exhibit reduced circadian rhythmicity, and studies have identified mechanisms that may underlie this phenomenon in specific contexts.[26–28] Melatonin can resynchronize some circadian rhythm genes in prostate cancer cells.[29] Research addressing the role of melatonin in tumor development in vivo is sparse, largely because the most commonly used inbred mouse strains do not synthesize melatonin due to genetic alterations that likely accumulated over generations of breeding in unnatural lighting conditions.[30] If melatonin plays a major role in suppressing tumor development as suggested by some studies,[31] the impact of circadian disruption on tumor

development may in fact be greatly underestimated in studies that rely on inbred mouse models.

3 | CRYPTOCHROMES ALTER DISEASE OUTCOMES IN MOUSE MODELS OF CANCER

Mouse models provide insight into the genetic determinants of cancer progression, and mice harboring mutations in core circadian clock components have been used to evaluate the potential for genetic disruption of circadian rhythms to alter tumor development.

A handful of studies have examined the impact of *Cry1* and *Cry2* deletion on tumor formation and survival in response to environmental exposures. Two groups measured overall survival in wildtype (WT) and $Cry1^{-/-}$;$Cry2^{-/-}$ mice following exposure to 4 Gy ionizing radiation delivered at zeitgeber time (ZT, hours after lights on) 10 to mice at either six or eight weeks of age,[32,33] respectively. One study reported increased tumor formation and decreased survival of $Cry1^{-/-}$; $Cry2^{-/-}$ mice compared to WT littermates following irradiation.[33] The other study[32] did not observe a significant difference in response to radiation in $Cry1^{-/-}$; $Cry2^{-/-}$ mice compared to WT mice of the same age and genetic background. It is unclear whether the different outcomes reflect the use of nonlittermate mice as controls in the 2005 study,[32] exposure to irradiation at different ages, or some other detail of the methodology or housing conditions. More recently, it was demonstrated that $Cry1^{-/-}$; $Cry2^{-/-}$ mice develop an increased tumor burden in response to diethylnitrosamine (DEN)-induced liver carcinogenesis.[34] Together, these reports support the hypothesis that cryptochromes reduce tumor formation in response to at least some environmental exposures. Consistent with these observations, $Cry1^{-/-}$;$Cry2^{-/-}$ mice exhibit decreased survival and form fewer but larger spontaneous hepatocellular carcinomas compared to WT mice.[13]

Several genetically engineered mouse models have been developed to mimic different types of cancer in humans. For example, TP53 is among the most widely inactivated human tumor suppressor genes and its deletion is used to accelerate the development of many tumor types in mice. Surprisingly, $Tp53^{-/-}$; $Cry1^{-/-}$; $Cry2^{-/-}$ mice have decreased tumor burden and increased overall survival compared to $Tp53^{-/-}$ mice[35] possibly due to differential transcriptome level changes in nuclear factor kappa B (NF-κB) regulation.[36] Thus, the effect of deleting *Cry1* and *Cry2* appears to impact tumor burden differently in different oncogenic settings. Specifically, this finding suggests that the impact of *Cry1* and/or *Cry2* deletion depends on the functional status of TP53.

Most studies that have investigated the role of cryptochromes in cancer have deleted *Cry1* and *Cry2* simultaneously (ie, $Cry1^{-/-}$; $Cry2^{-/-}$ mice), presuming their functions to be redundant. However, while either CRY1 or CRY2 can support circadian rhythmicity[37,38] subsequent studies have demonstrated that their functions are divergent both within[37,39] and especially outside of[40–43] the circadian clock mechanism. Therefore, it is important to study their potential roles in tumor formation separately. $Cry2^{-/-}$ mice and mice deficient for any three of the four *Cry1* and *Cry2* alleles in a $Tp53^{-/-}$ background exhibited increased survival

compared to *Tp53*$^{-/-}$ mice[35] while deletion of *Cry2* increased tumor burden and decreased overall survival in a MYC-driven lymphoma mouse model.[42]

Chronic inflammation can promote tumor formation at affected sites.[6] Altering circadian rhythms has been shown to impact immune responses.[44] *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ mice display constitutive elevation of proinflammatory cytokines[45–47] and an autoimmune phenotype.[45,48] The proinflammatory phenotype of *Cry1*$^{-/-}$; *Cry2*$^{-/-}$ mice may contribute to increased cancer formation upon irradiation.[33]

Together, these findings indicate that inactivation of cryptochromes impacts the development of a variety of cancers in mice. Furthermore, the effect of deleting *Cry1* or *Cry2* depends on the tissue of origin and on the underlying genetic drivers of tumorigenesis. Finally, CRY1 and CRY2 have unique functions in regulating cancer development and must be studied independently.

4 | CONNECTING CRYPTOCHROMES WITH TUMOR SUPPRESSORS

To sustain proliferation, tumor cells must overcome growth-inhibitory signals such as cell cycle inhibition via tumor suppressor genes. Recent reviews have described several molecular connections between cell cycle inhibition and circadian proteins.[49,50] In this section, we focus on evidence suggesting that cryptochromes can interact with or influence widely known tumor suppressor pathways.

4.1 | TP53

Tumor suppressor protein 53 (TP53) is the most frequently inactivated tumor suppressor in human cancer.[6] In normal cells, it is activated by intracellular stresses, such as DNA damage and reactive oxygen species, leading to cell cycle arrest and/or apoptosis.[6] Thus, its inactivation allows damaged cells to survive and expand. *TP53* expression and/or TP53 phosphorylation exhibit daily oscillations in human oral mucosal cells[51] and mouse thymus.[33] Furthermore, TP53 protein exhibits a circadian rhythm in fibroblast-like cells either implanted in mice or subject to circadian synchronization in culture, while *Tp53* mRNA was expressed at a constant level, which was attributed to rhythmic ATF4-driven repression of *p19* interfering with MDM2-dependent TP53 destabilization.[52] This suggests that TP53 protein levels are subject to posttranscriptional regulation by circadian clocks. Several studies have suggested that clock proteins can modulate the function of TP53.

TP53 stability is regulated by the E3 ubiquitin ligases mouse double minute 2 homolog (MDM2)[53–55] and constitutive photomorphogenic 1 (COP1)[56] and the ubiquitin-specific protease herpesvirus-associated ubiquitin-specific protease (HAUSP) that removes polyubiquitin chains from TP53[57,58] (Figure 1). Upon DNA damage, HAUSP can deubiquitinate CRY1 to promote CRY1 stabilization.[40] Whether CRY1 has any effect on TP53 function or stability mediated by its interaction with HAUSP remains unclear. DNA damage was also shown to decrease CRY2 stability by increasing the interaction between CRY2 and the Skp-Cullin-Fbox (SCF) E3 ubiquitin ligase substrate receptor F-box and leucine-rich repeat protein 3 (FBXL3).[59–61] Both CRY1 and CRY2 can interact with De-etiolated1 (DET1), thus inhibiting recruitment of the substrate receptor COP1 to the Cullin 4

(CUL4)-based E3 ubiquitin ligase complex.[62] This could result in CRY1/2-dependent TP53 stabilization as COP1 is a negative regulator of TP53[56] (Figure 1). Consistent with this possibility, depletion of *CRY1* in osteosarcoma cells decreased TP53 and P21 levels and increased proliferation and migration.[63] Conversely, *Cry1*-deficient primary fibroblasts exhibit prolonged activation of *P21* transcription in response to DNA damage, while loss of CRY2 suppressed *P21* expression in the same context.[40] Additionally, shRNA-mediated reduction of *Tp53* results in increased cell proliferation and growth in low paracrine signaling conditions in $Cry2^{-/-}$ mouse embryonic fibroblasts (MEFs) compared to WT cells, while $Cry1^{-/-}$ MEFs grow more slowly than WT cells in response to depletion of TP53.[42]

PERs and CRYs function as heterodimers to repress CLOCK- and BMAL1-driven transcription in the TTFL. PER2 can bind the C-terminal region of TP53 to prevent MDM2-mediated degradation, thereby promoting TP53 stabilization and increasing TP53-mediated target gene expression.[64–66] Cryptochromes might influence these actions given their robust interactions with PER2. The impacts of cell type, stimulus context, and other factors in the role of cryptochromes in TP53 regulation remain to be determined.

4.2 | RB

Inactivation of RB causes childhood retinoblastoma. RB suppresses cell growth by preventing adenoviral early region 2 binding factor (E2F) from activating the expression of cell cycle promoting genes.[67] Phosphorylation of RB by cyclin-dependent kinases 4 and 6 (CDK4/6) prevents its interaction with E2Fs, and CDK4/6 inhibitors are effective in treating cancers with intact RB function.[67] CDK4/6 protein and RB phosphorylation were enhanced by treatments that mimic chronic circadian disruption in human osteosarcoma cells (U2OS).[68] This phenomenon was blocked by genetic depletion of either *Bmal1* or of both *Cry1* and *Cry2*. We recently discovered an interaction of both CRY1 and CRY2 with several E2F family transcription factors. While only CRY2 stimulates the ubiquitination and turnover of c-MYC by recruiting it to SCF^{FBXL3},[42] we found that both CRY1 and CRY2 can interact with E2F family members and stimulate their interaction with FBXL3[69] (Figure 1). Steady-state levels of E2F4 and E2F8 are robustly affected by modulating *Cry1* and/or *Cry2* expression, but E2F1 protein levels are less susceptible to modulation by cryptochromes. CRY1 and CRY2 may influence E2F-dependent transcription through direct repression, but this requires further investigation.

4.3 | LKB1

Peutz-Jeghers syndrome is an inherited cancer susceptibility caused by inactivating mutations in liver kinase B1 (LKB1).[70] LKB1 phosphorylates and thereby activates a family of fourteen related kinases that includes adenosine monophosphate–activated protein kinase (AMPK).[71] Recent work demonstrated that the tumor suppressor function of LKB1 depends on activation of a subfamily of four AMPK-related kinases, the microtubule affinity regulating kinases (MARK1–4).[72] AMPK phosphorylates CRY1 on two serines (S71 and S280) (Figure 1), and thereby disrupts its interaction with PER2[73] and promotes CRY1 association with FBXL3, leading to CRY1 ubiquitination and degradation[18] (Figure 1). All of the LKB1-activated kinases are expected to phosphorylate substrates containing similar

amino acid sequences as those phosphorylated by AMPK, but it is unclear whether MARKs can phosphorylate CRY1 or CRY2.

AMPK is a master regulator of metabolic function and is a target of the widely used diabetes drug, metformin,[71] which is currently being evaluated for therapeutic efficacy in cancer.[74] A recent study examined the relationship between expression of clock genes and sensitivity to a variety of established or proposed chemotherapeutic agents. The drugs tested include two that activate AMPK (AICAR and phenformin). Intriguingly, among the fourteen core clock genes examined, only the expression of *CRY1* is positively correlated and of *PER2* is negatively correlated with sensitivity to both drugs,[8] suggesting that AMPK-dependent disruption of CRY1-PER2 interaction may influence cell viability. Although neither the transport and metabolism of metformin, nor the expression of AMPK catalytic subunits are affected by the time of day, activation of AMPK in the liver by equivalent doses of metformin is much greater during the active phase in mice,[75] suggesting that timing of metformin delivery may influence its efficacy in diabetes and/or cancer treatment.

4.4 | DNA damage–responsive kinases

Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are the central kinases that initiate the DNA damage response in cells. ATM is activated by double-strand breaks, while ATR responds to stalled DNA replication forks.[76] TIMELESS (TIM) is a large intrinsically disordered protein that is required for circadian rhythms in *Drosophila melanogaster*.[77] The role of mammalian TIM in circadian rhythms has been difficult to study due to its essential function in embryonic survival.[78] Human *TIM* mutants cause advanced sleep phase,[79] suggesting that TIM also influences the mammalian clock,[80,81] an idea that is also supported by protein-protein interactions between mammalian TIM, CRY1,[81,82] and CRY2.[83] TIM has been shown to facilitate the ATR replication checkpoint,[84] and CRY1 can participate in the ATR-mediated DNA damage checkpoint via interaction with TIM[82] (Figure 1).

Following DNA damage, DNA-dependent protein kinase (DNA-PK) can phosphorylate the C-terminal tail of CRY1 at Serine 588[40,85] (Figure 1). It is unclear whether or how this phosphorylation impacts the interaction of CRY1 with TIM and/or ATR-mediated signaling. Furthermore, TIM has a role in genome integrity through facilitating sister chromatid cohesion.[86] Consequently, alterations in CRY1 or CRY2 interaction could result in aberrant mitosis and disrupted genome integrity.

Though less well studied than the glutamine-directed protein kinases that are activated by DNA damage (ATM, ATR, and DNA-PK), the mammalian tousled-like kinases, TLK1 and TLK2, are maximally active in dividing cells at the time of peak DNA replication and are further activated in response to treatment with a variety of DNA-damaging agents during S phase.[87] TLK2 is frequently amplified in estrogen receptor–positive breast tumors and its overexpression enhanced aggressive growth of breast cancer–derived cell lines.[88] CRY1 and CRY2 can recruit TLK2 to FBXL3, and genetic deletion of *Cry1* and *Cry2* increased the steady-state protein level of inducibly expressed TLK2 in primary fibroblasts.[89]

5 | CRY-ING OVER ONCOGENES

To become malignant, cells must replicate indefinitely.[6] This can be achieved through overactivation of pro-growth pathways or deregulation of the cell cycle. The circadian and cell cycles influence each other,[90–94] and several molecular connections between the two have been described. Cryptochromes impact both expression and posttranslational regulation of established cell cycle regulators. Deletion of *Cry1* and/or *Cry2* alters the expression of several genes involved in cell cycle regulation, including *Cyclin D1*, *Wee1*, *Cyclin A2*, and *Cyclin B1*,[90] and affects the steady-state protein levels of c-MYC[42] and of several members of the E2F family of transcription factors (E2Fs).[69] A recent study developed a method to approximate chronic circadian disruption in cultured cells and demonstrated that it increases proliferation. The enhanced proliferation caused by circadian disruption was prevented by depletion of *Cry1* and *Cry2*,[68] suggesting that cryptochromes may play an important role in linking circadian disruption to enhanced cancer risk.

5.1 | MYC

c-MYC is among the most highly amplified oncogenes in human cancer.[95] c-MYC is an important regulator of cell proliferation, transcription, differentiation, apoptosis, and cell migration.[96] In primary MEFs, *c-MYC* stable overexpression leads to increased proliferation and growth in low paracrine signaling conditions; these effects were greatly enhanced in *Cry2*$^{-/-}$ compared to WT or *Cry1*$^{-/-}$ MEFs.[42] Similar to the well-established regulation of c-MYC by FBXW7,[97] CRY2 cooperates with FBXL3 to interact with c-MYC and promote its ubiquitination and degradation.[42] Intriguingly, not only does DNA damage enhance the interaction between FBXL3 and CRY2,[40] but FBXL3 was one of only four F-box substrate adaptors that exhibited significantly enhanced recruitment to CUL1 in response to DNA damage in a recent study.[98] In line with this finding, knockdown of *CRY2* in osteosarcoma cells increased c-MYC and decreased TP53 protein levels.[99] *Fbxw7* deletion in either T cells or hematopoietic stem cells can cause thymic lymphoma[100] or acute lymphoblastic leukemia,[101] respectively. These effects of *Fbxw7* deletion are accelerated in *Tp53*$^{-/-}$, *Pten* $^{-/-}$, or NOTCH activation mouse models.[102] Germline deletion of *Cry2* in the *Eμ-Myc* lymphoma mouse model increased tumor burden and decreased overall survival.[42] Additional studies are needed to understand whether *Cry2* deletion has a cell-autonomous effect on tumor development and to elucidate the context-dependent roles of *Cry2* deletion in tumor development in vivo. Conversely, MYC opposes BMAL1 expression and/or activity and thus dampens circadian rhythms.[26,103,104] Reciprocal regulation of circadian rhythms and MYC further highlights the dynamic interaction between circadian and cell cycle oscillations.

5.2 | RAS

In the early days of oncogene discovery, several tumor-inducing retroviruses that cause the formation of sarcomas in rats were found to promote tumorigenesis by way of a common viral gene. This so-called rat sarcoma (*ras*) viral oncogene has several mammalian orthologs —encoded by the Kirsten (*K*), Harvey (*H*), and neuroblastoma (*N*) *ras* oncogenes. The protein products of *Kras*, *Hras*, and *Nras,* collectively referred to as RAS, are activated by growth factor receptors and hydrolyze guanosine triphosphate (GTP) to promote the

activation of downstream signaling pathways, including phosphatidylinositol 3-kinases (PI3Ks) and mitogen-activated protein kinases (MAPKs). RAS proteins are the most frequent targets of mutation in cancer, with mutations that prevent their association with GTPase-activating proteins (GAPs; eg, *HRas*^{G12V} a.k.a *HRas*^{V12}) having the most powerful influence on cellular transformation.

HRas^{V12} overexpression lengthens circadian period and decreases *Cry1* expression in MEFs.[27,105] MEFs generated from *Bmal1*$^{-/-}$ or *Clock* mutant mice are resistant to oncogenic transformation by combined overexpression of *H-Ras*^{V12} and SV40 large T antigen (*SV40LT*). This is likely due to their inability to activate transcription of *Atf4* and thereby repress the tumor suppressor *p19Arf*, such that p19ARF maintains its suppression of MDM2-mediated TP53 degradation.[106] In the absence of SV40LT co-expression, depletion of *Bmal1* had no impact on proliferation or senescence in MEFs overexpressing *H-Ras*^{V12}.[105] MEFs deficient in period or cryptochrome repressors expressed normal levels of ATF4 and were transformed by *HRas*^{V12} to a similar extent as WT cells. This difference in transformation could reflect reduced expression of genes directly controlled by CLOCK and BMAL1 in *Bmal1* or *Clock* mutants, while loss of *Per2,* or *Cry1* and *Cry2*, may result in elevated expression of those genes either constitutively or with an enhanced amplitude of rhythmicity. Finally, *Cry2*$^{-/-}$ MEFs were more susceptible to *H-Ras*^{V12} transformation than WT or *Cry1*$^{-/-}$ MEFs.[42] These studies suggest a possible CRY2-specific effect on RAS-dependent signaling; it is unclear whether this is mediated by effects on ATF4, c-MYC, core clock function, or some other mechanism. Like c-MYC, RAS has been shown to reciprocally affect the circadian clock.[27]

6 | EXPRESSION PATTERNS AND POLYMORPHISMS OF *CRY1* AND *CRY2* IN HUMAN CANCERS

Many studies have demonstrated that long-term shift work increases the relative risk of several types of cancer.[107–114] Even the longitudinal position within a time zone at which a person resides significantly impacts cancer risk.[115,116] Together, these findings suggest that chronic alterations in circadian environmental exposures alter tumor development. Association studies have identified single nucleotide polymorphisms (SNPs) in *CRY*s that associate with risk or mortality for various cancers, including prostate cancer,[117–119] non-Hodgkin's lymphoma,[120] and breast cancer.[121] Further research is needed to determine whether these SNPs are reproducibly associated with tumor development in additional populations. Although amplification, deletion, and missense mutation of core clock genes are rare in cancer, several studies have measured significantly altered expression of *CRY1* or *CRY2* in a variety of cancer types (Table 1). Generally, there seems to be a strong tendency toward decreased expression of *CRY1* and especially of *CRY2* in a variety of human cancers. Furthermore, *CRY2* expression has a much greater tendency than that of *CRY1* to be associated with altered activity of established oncogenic or tumor suppressive pathways.[8] This suggests that CRY2 could have a more dominant role than CRY1 in altering cancer-relevant signaling pathways. Notably, these analyses used exome sequencing data compiled in The Cancer Genome Atlas (TCGA)[122]; the recently released Pan-cancer analysis of whole genomes (PCAWG)[123] will enable investigation of noncoding variants.

Tumors tend to have reduced or absent circadian rhythmicity either due to individual oncogenes,[26,27] proteins up-regulated by oncogenic transformation,[28] or lack of access to peripheral synchronizing factors.[124] It is unclear whether the loss of rhythmicity could confound measurements of gene expression. Furthermore, most of these studies only examined *CRY1/2* expression and did not investigate whether these tumors maintained circadian rhythmicity. Typically, tumor resection was performed between 9:00 AM and 5:00 PM, but few studies precisely record the time of resection. In addition, lighting conditions are usually not well described. These factors could influence *CRY1/2* expression, and the lack of such information complicates the interpretation of data from samples for which they are not recorded.

7 | CRYPTOCHROMES MAY IMPACT DRUG EFFICACY

The idea of chronotherapy considers the time of day when the therapy is dosed to improve efficacy or to avert negative side effects or toxicity of the therapy.[133] This idea suggests that the circadian clock and its core components most likely play a role in modulating therapy outcomes.

Cry1$^{-/-}$; *Cry2*$^{-/-}$ mice showed less toxic side effects and were more resistant toward the anticancer drug, cyclophosphamide, compared to their WT littermates. This effect was not due to changes in cyclophosphamide metabolism and/ or detoxification rates,[134] although cryptochromes have been shown to suppress other drug metabolism pathways.[135] Whether chemoresistance to cyclophosphamide in *Cry1*$^{-/-}$; *Cry2*$^{-/-}$ mice compared to WT littermates applies in a cancer mouse model or xenograft model will be an interesting area for further study.

Platinum-based chemotherapeutic agents, including cisplatin and oxaliplatin, drive cytotoxicity in dividing cells by forming adducts with nuclear DNA, and thus causing DNA damage and interfering with replication and transcription. Cryptochromes are evolutionarily related to bacterial DNA repair enzymes known as photolyases.[16] While mammalian CRY1 and CRY2 lack DNA repair activity, daily oscillations in the transcript and protein levels[136] of the nucleotide excision repair enzyme xeroderma pigmentosum group A (XPA) have been documented in livers extracted from mice. Furthermore, XPA and nucleotide excision repair activity are elevated in *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ livers.[136] Thus, cryptochromes could suppress DNA repair in response to platinum-based chemotherapeutic treatments; if so, the timing of treatment delivery could impact its effectiveness and/ or toxicity.

In addition to modulating DNA repair activity through XPA expression, CRY1 and CRY2 can influence the cellular response to DNA damage. The apoptotic effects of oxaliplatin in RAS-transformed, *Tp53*$^{-/-}$ cells are increased by deletion of *Cry1* and *Cry2* due to increased expression of *Egr1* and *p73*, but the apoptotic effects of doxorubicin are not impacted by *Cry* mutation.[137] Consistent with the indication that CRY1 and/or CRY2 oppose the cytotoxic effect of oxaliplatin, chemoresistant colon cancer samples were found to have high expression of *CRY2* and depletion of *CRY2* rendered them sensitive to oxaliplatin.[138] Furthermore, *Tp53*$^{-/-}$; *Cry1*$^{-/-}$; *Cry2*$^{-/-}$ xenografts were reportedly more sensitive to oxaliplatin treatment than *Tp53*$^{-/-}$ xenografts, due to increased expression of p73 leading to

increased apoptosis in response to oxaliplatin.[139] Together, these findings suggest that cryptochromes impact the efficacy of platinum-based chemotherapeutic agents. Additional investigations will be required to understand why their impact on the response to other DNA-damaging agents like doxorubicin seems to be different.

The xenobiotic receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are members of the nuclear hormone receptor (NR) superfamily of transcription factors.[140] PXR and CAR are activated by a wide variety of lipophilic ligands including many drugs and environmental toxins. Cryptochromes interact with many NRs, including PXR and CAR, and suppress NR-driven transcriptional activation.[46,135,141] Because PXR and CAR activate transcriptional networks that stimulate drug transport, activation, and metabolism, this suggests that circadian modulation of their activity by cryptochromes could alter the effectiveness and/or toxicity of clinically useful compounds in a time-of-day-dependent manner. In addition, several cancers are influenced by NR actions. Additional studies are needed to reveal whether and how cryptochromes and NRs function together to influence outcomes in hormone-dependent cancers, like breast and prostate cancers, in which estrogen, progesterone, and androgen receptors' expression and/or mutation play an important role.[142–144]

8 | CHEMICAL COMPOUNDS THAT DIRECTLY TARGET CRYPTOCHROMES

In addition to the potential for cryptochromes to alter the pharmacokinetics or efficacy of other drugs, selective modulation of cryptochromes themselves may be therapeutic. A handful of compounds that stabilize or inhibit cryptochromes have been described. The first such small molecule identified (KL001) stabilizes both CRY1 and CRY2[145] by inhibiting their interaction with FBXL3.[146] KL001 suppresses gluconeogenesis in hepatocytes, suggesting that pharmacological targeting of cryptochromes could be useful in metabolic disease.[145,147] Many CRY-stabilizing derivatives have been made,[148–150] and it was recently demonstrated that both KL001 and the derivative SHP656 selectively kill glioblastoma stem cells in vitro and in vivo.[150]

KS15 is a small molecule that inhibits CRY1- and CRY2-dependent repression of CLOCK and BMAL1 without affecting CRY1/2 stability.[151]Treatment of breast cancer cells in vitro with high concentrations of KS15 reduced their proliferation and increased their sensitivity to doxorubicin.[152] It is unclear whether these reported effects of KS15 require its regulation of CRY1 and/or CRY2.

9 | CONCLUSIONS AND OUTLOOK

Cryptochromes participate in a wide variety of cellular functions that may influence cancer growth in addition to generating circadian rhythms. While we focused on cryptochromes in this review, there are likely multiple mechanisms by which circadian clocks influence the cell cycle and cancer. Alteration in the expression of *Cry* or any other core clock gene undoubtedly will affect expression of other circadian proteins and their downstream targets, making it difficult to delineate the direct and indirect impacts of any perturbation of the

clock. CRY1 and CRY2 are often thought to have similar functions, but it is imperative that they are investigated as separate entities because they clearly have several differentiated roles. Also, cryptochromes can function differently in different cellular pathways depending on context. Further development of cryptochromes as therapeutic targets in cancer or other diseases will require additional clinical and structure-function relationship studies. Circadian rhythms are pervasive and influence many of the widely accepted cancer hallmarks.[6,11,153] Incorporating a better understanding of the role of circadian rhythms and time of day in basic and clinical cancer research will improve evidence-based recommendations for public health and clinical practice.

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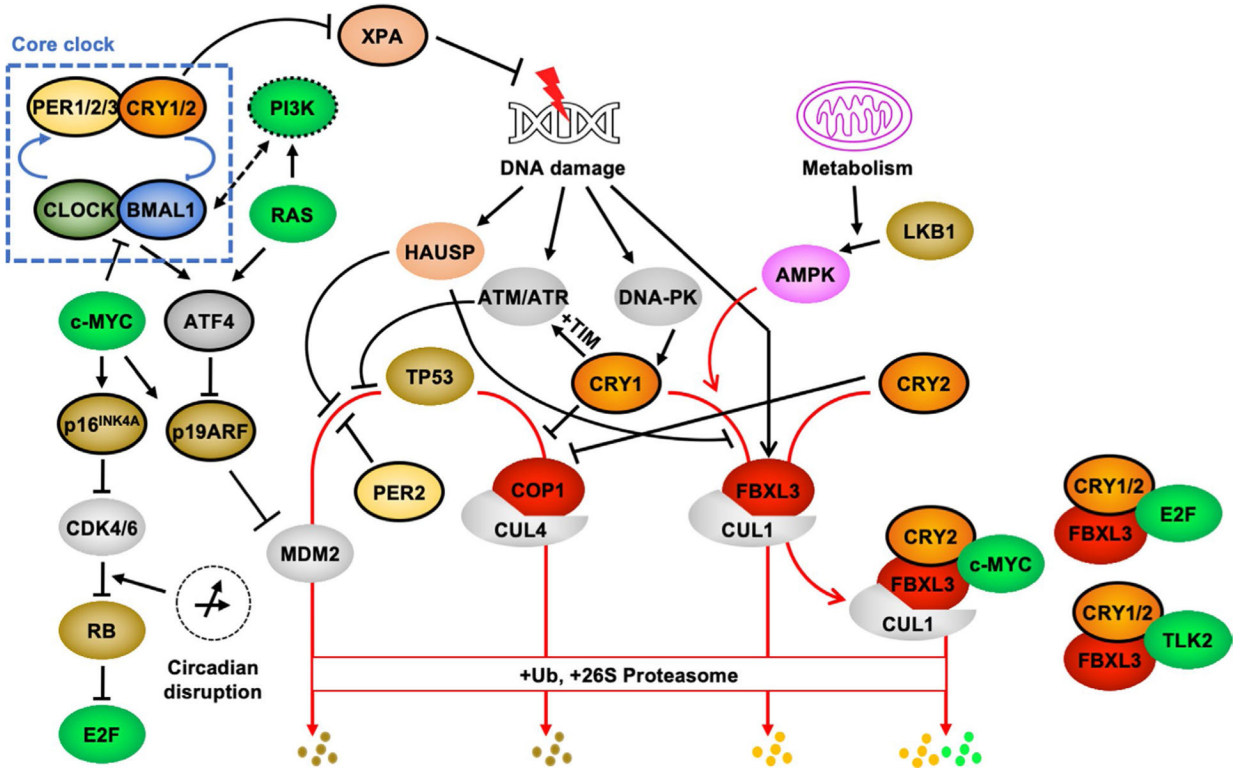

FIGURE 1.

Molecular connections between CRY1, CRY2, and cancer-related pathways. The core clock mechanism involves a transcription-translation feedback loop (TTFL) in which the bHLH-PAS transcription factors CLOCK and BMAL1 drive expression of their own repressors, PERs and CRYs (for a more complete description of the molecular clock, see ref.[1]). In the face of growing interest in understanding the mechanistic underpinnings for enhanced cancer risk in people exposed to chronic circadian disruption, several molecular connections between clocks and proteins with established roles in regulating cell growth, DNA repair, and the cellular response to DNA damage have been described. See text for details. Proteins outlined in black exhibit widespread circadian expression at the mRNA level. Brown shading denotes tumor suppressors; bright green shading denotes oncogenes. Substrate receptors for multisubunit E3 ligases are shown in red. Red arrows indicate pathways that lead to ubiquitination and 26S proteasome-mediated degradation. Ub, ubiquitin

TABLE 1

Correlation of *CRY1* and/ or *CRY2* expression with human tumor phenotype (vs normal control tissue) and with survival outcome

Cancer type	*CRY1*	*CRY2*	References
Gastric	Increased; associated with advanced stage NC	NC Decreased	125 8
Thyroid	NC Decreased	Decreased Decreased	126 8
Glioma	NC	Increased Associated with poor outcome	127 8
Skin	Decreased NC	Decreased	128 42
Ovarian	Increased; associated with improved survival	Decreased	129
Pancreas (PDAC)	Decreased	Decreased	130
Colorectal	NC Decreased NC	Decreased Decreased Decreased	131 132 8
Lung	Decreased (LUSC and LUAD) Decreased	Decreased (LUSC and LUAD) Decreased	8 42
Kidney	Decreased in KICH	Increased (KICH), Decreased (KIRP); low expression associated with poor outcomes (KIRP, KIRC)	8
Breast	NC NC	Decreased; alters subtype Decreased	8 42
Liver	NC	NC; low expression associated with worse outcome	8
Head and Neck	NC	Decreased	8
Bladder	NC	Decreased	8
Esophagus	Decreased	Decreased	8
Prostate	NC	NC	8
Bone	NC	Decreased	42

Abbreviations: KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NC, no change; PDAC, pancreatic ductal adenocarcinoma.