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A Role for the Circadian Clock in Colorectal Cancer Progression: A Comprehensive Molecular Analysis on the Interplay between Core-Clock Genes and Metastasis-related Cellular Processes

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Zusammenfassung

Störungen der zirkadianen Uhr haben weitreichende Auswirkungen auf verschiedene zelluläre Prozesse, einschließlich Transkription, Zellzyklus, Stoffwechsel und letztlich Homöostase. Solche Störungen können die abnormale Regulation zellulärer Eigenschaften auslösen und die Tumorentstehung stimulieren. In unseren Studien untersuchten wir die Auswirkungen von Störungen der zirkadianen Uhr auf Krebsentwicklung durch die Herunterregulation oder das Ausschalten von Kern-Uhrgenen wie *BMAL1* (auch bekannt als *ARNTL*), *PER2* oder *NR1D1*. Wir untersuchten dies sowohl *in vitro* als auch in *in vivo* Xenotransplantationsmodellen, wobei wir uns auf die Darmkrebszelllinien HCT116, SW480 und SW620 konzentrierten. Wir entdeckten, dass diese Manipulationen die zirkadiane Expression von Schlüsselgenen wie *MYC*, *WEE1* und *TP53* (die an der Regulierung des Zellzyklus und der Apoptose beteiligt sind) sowie *MACC1* (das mit der epithelial-mesenchymalen Transition (EMT) zusammenhängt und für seine Rolle bei der Metastasierung bekannt ist) beeinflussen, wodurch wichtige zelluläre Eigenschaften wie Proliferation, Apoptose, Zellmigration und Invasion moduliert werden. Bemerkenswert ist, dass das Ausschalten von *NR1D1* eine Beeinträchtigung der Zellbeweglichkeit *in vitro* und eine Reduktion der Mikrometastasenbildung *in vivo* zur Folge hatte. Dies ging mit veränderten Expressionsniveaus wesentlicher EMT-assoziierter Gene, *SNAI1* und *CD44*, einher.

Wir konnten außerdem zeigen, dass das MACC1-Protein in HCT116 Wildtyp-Zellen zirkadian (mit einer Periode von etwa 24 Stunden) exprimiert wurde, eine Regulierung, die nach dem Ausschalten der erwähnten Kern-Uhrgene gestört war. Darüber hinaus identifizierten wir Protein-Protein-Interaktionen zwischen MACC1 und NR1D1, was

eine potenziell neue Achse der Regulation in der Progression des Darmkrebses über zirkadiane Uhrelemente darstellt. Interessanterweise demonstrierten wir die Auswirkungen der MACC1-Manipulation, entweder durch Ausschalten oder Überexpression von *MACC1*, auf das Phänotyp der zirkadianen Uhr und den Krebsfortschritt in Darmkrebszellen. Wir fanden heraus, dass das Ausschalten von *MACC1* die Periode der *BMAL1*-Promotor-Oszillationen verringerte, während seine Überexpression den gegenteiligen Effekt in Darmkrebszellen hatte. Dieses bidirektionale Zusammenspiel zwischen *MACC1* und der zirkadianen Uhr weist auf die Komplexität der Mechanismen im Darmkrebs hin und betont die Rolle der zirkadianen Uhr bei der Regulation der Darmkrebs-Metastasierung.

Zusammenfassend unterstreichen die Ergebnisse unserer Studien die bedeutende Rolle von Kern-Uhrgenen bei wichtigen Krebseigenschaften. Dazu gehören die Regulation von Zellmigration, Zellinvasion und das komplexe Zusammenspiel von *MACC1* und zirkadianer Uhr in Darmkrebs, das möglicherweise durch Interaktionen mit Kern-Uhrgenen vermittelt wird. Unsere Daten bieten tiefere Einblicke in das molekulare Zusammenspiel zwischen der zirkadianen Uhr und Krebs und weisen auf einen komplexen, aber vielversprechenden Bereich für therapeutische Interventionen hin. Künftige Forschungsarbeiten können die Entwicklung chronotherapeutischer Strategien untersuchen, die unser Verständnis der zirkadianen Kontrolle der Proliferation, Invasion und Metastase von Krebszellen nutzen. Dieser Ansatz könnte wirksamere und personalisierte Krebstherapien ermöglichen, indem therapeutische Interventionen auf individuelle zirkadiane Rhythmen abgestimmt werden, um ihre Wirksamkeit zu verbessern und Nebenwirkungen zu verringern. Darüber hinaus ermöglichen die identifizierten Wechselwirkungen zwischen Komponenten der zirkadianen Uhr und Krebsmechanismen eine weitere Untersuchung der molekularen

Mechanismen, die diesen Verbindungen zugrunde liegen, was zur Entdeckung neuer Biomarker und therapeutischer Targets bei Darmkrebs führen könnte.

Schlagwörter:

Zirkadiane Uhr, Darmkrebs, MACC1, Zellinvasion

Abstract

Circadian clock disruptions have far-reaching impacts on several cellular processes, including transcription, cell cycle, metabolism and ultimately homeostasis. Such disturbances can trigger the abnormal regulation of cellular properties and stimulate tumorigenesis. In our studies, we aimed to investigate the effects on cancer progression of disruptions in the circadian clock caused by the downregulation or knockout of core-clock genes *BMAL1* (aka *ARNTL*), *PER2* or *NR1D1*. We explored this using both *in vitro* and *in vivo* xenograft models, utilizing colorectal cancer (CRC) cell lines HCT116, SW480 and SW620. We discovered that these manipulations affect the circadian expression of key genes such as *MYC*, *WEE1* and *TP53* (involved in cell cycle regulation and apoptosis) as well as *MACC1* (related to epithelial-mesenchymal transition, EMT, and known for its role in driving metastasis), subsequently modulating key cellular properties such as proliferation, apoptosis, cell migration, and invasion. Notably, the knockdown of *NR1D1* led to reduced cell motility *in vitro* and a decrease in micrometastasis formation *in vivo*. This coincided with modified expression levels of essential EMT-associated genes, *SNAI1* and *CD44*.

We further revealed that the MACC1 protein is circadian expressed (with a period of about 24 hours) in HCT116 WT cells, a regulation that was disrupted following the knockout of mentioned core-clock genes. In addition, we identified protein–protein interaction between MACC1 and NR1D1, illustrating a potential new axis of regulation in CRC progression via circadian clock elements. Remarkably, our data illustrate that modulation of *MACC1*, via either gene knockout or overexpression, directly impacts the circadian clock phenotype and the subsequent progression of cancer cells. We found that *MACC1* knockout reduced the period of *BMAL1*-promoter oscillations, while

its overexpression had the opposite effect in CRC cell lines. This bidirectional interplay between *MACC1* and the circadian clock points to the complexity of the mechanisms at play in CRC and highlights a role for the circadian clock in regulating CRC metastasis.

In conclusion, the results of our studies underline the significant roles of core-clock genes in key cancer properties. These include the regulation of cell migration, invasion, and the intricate *MACC1*-circadian clock interplay in CRC, potentially mediated through interaction with core-clock genes. Our data provide deeper insights into the molecular interplay between the circadian clock and cancer progression, pointing to a complex, yet promising area of therapeutic intervention. Future research could explore the development of chronotherapeutic strategies, leveraging our understanding of the circadian regulation of cancer cell proliferation, invasion, and metastasis. This approach may offer more effective and personalized cancer treatments by aligning therapeutic interventions with individual circadian rhythms, thereby enhancing efficacy and reducing side effects. Additionally, the identified interactions between circadian clock components and cancer pathways enable further investigation into the molecular mechanisms underlying these links, potentially leading to the discovery of novel biomarkers and therapeutic targets in colorectal cancer.

Keywords:

Circadian Clock, Colorectal Cancer, MACC1, Cell Invasion

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List of Abbreviations

3D	Three Dimensional
CCG	Clock-Controlled Gene
CCN	Core-Clock Network
CRC	Colorectal Cancer
dpi	Days Post-Injection
EMT	Epithelial-Mesenchymal Transition
hpf	Hours Post-Fertilisation
KD	Knockdown
KO	Knockout
SCN	Suprachiasmatic Nucleus

1 Introduction

1.1 The Circadian Clock System

Earth's daily rotation cycle generates certain behavioural patterns in organisms around the world. The ability to coordinate and synchronize to temporal niches and to the external environment is governed by an endogenous circadian clock in all organisms. These environmental changes (light-dark cycles, sleep-activity cycles and eating-fasting cycles) drive daily rhythms in behaviour, physiology and metabolism by interacting with the endogenous circadian clocks. A defining characteristic of circadian rhythms is their ability to persist even without external environmental signals. Furthermore, they offer organisms a competitive edge by enabling more efficient energy management and bolstering their resilience to oxidative stress cycles associated with respiration [1]. Altogether, circadian rhythms allow the organism to fine-tune its physiological and behavioural pattern to the diverse external conditions in a proactive rather than a responsive manner (reviewed in [2]).

The significance of circadian regulation was recently explored in primates and mice. Analysis of the circadian transcriptome showed that a vast majority of the primate and mouse genomes undergoes daily rhythmic variations in gene transcription/expression that are tissue- and locus-specific [3,4]. These rhythmic variations govern many critical cellular and tissue functions, holding substantial relevance for public health strategies, disease prevention, and disease management protocols. In humans, the circadian rhythmicity, which spans approximately 24 hours in length, regulates several biological and physiological processes. From molecular processes such as cell division, cell cycle progression and DNA repair [5-8], to physiological modifications in body temperature, blood pressure and circulating hormones [9,10], as well as greater cellular pathways, including splicing, RNA transport, cancer related pathways, cellular

metabolism and the immune system [11-15], all are under circadian regulation. For humans, the importance of a healthy endogenous clock has been well demonstrated in several epidemiological studies. Circadian desynchronizations to the environment (e.g., by shift work) show higher incidences of several types of cancer. Some examples include non-Hodgkin's lymphoma, breast, endometrial, prostate and colon cancers, wherein the magnitude of the impact correlates with the extent and intensity of circadian perturbations (reviewed in [16]). From disruptions in metabolic pathways [13], to dysregulations in splicing and cell cycle [14,17-20] and impairments in the immune system [15], all show severe consequences of circadian clock malfunction in a cancer context. These and other findings have prompted the World Health Organization to classify circadian disruptions as a potential carcinogenic risk for humans [21].

1.2 The Central Clock

In mammals, the suprachiasmatic nucleus (SCN), located at the base of the anterior hypothalamus above the optic chiasm, is considered the master pacemaker of the circadian system in the entire body. Neurons in the SCN obtain photic information from the retina through synaptic communication from axons of the retinohypothalamic tract. This electrical data transform into chemical signals, modifying the phase of clock gene expression within a specific group of SCN neurons. The SCN then conveys its rhythmic information to cells in other brain areas and peripheral organs through several pathways, encompassing neuronal links, hormonal signals, temperature cycle fluctuations, and secondary cues stemming from oscillating behaviour **(Figure 1)** [22]. It is important to mention that the SCN should be more accurately considered the "master synchronizer" rather than a strict pacemaker, since most tissues and cell types exhibit circadian patterns of gene expression even after being removed from the SCN [23-25]. Consequently, the SCN is believed to function as the central orchestrator of

an ensemble of biological oscillators, harmonizing all intrinsic clocks and integrating peripheral data to produce coordinated systemic circadian rhythms.

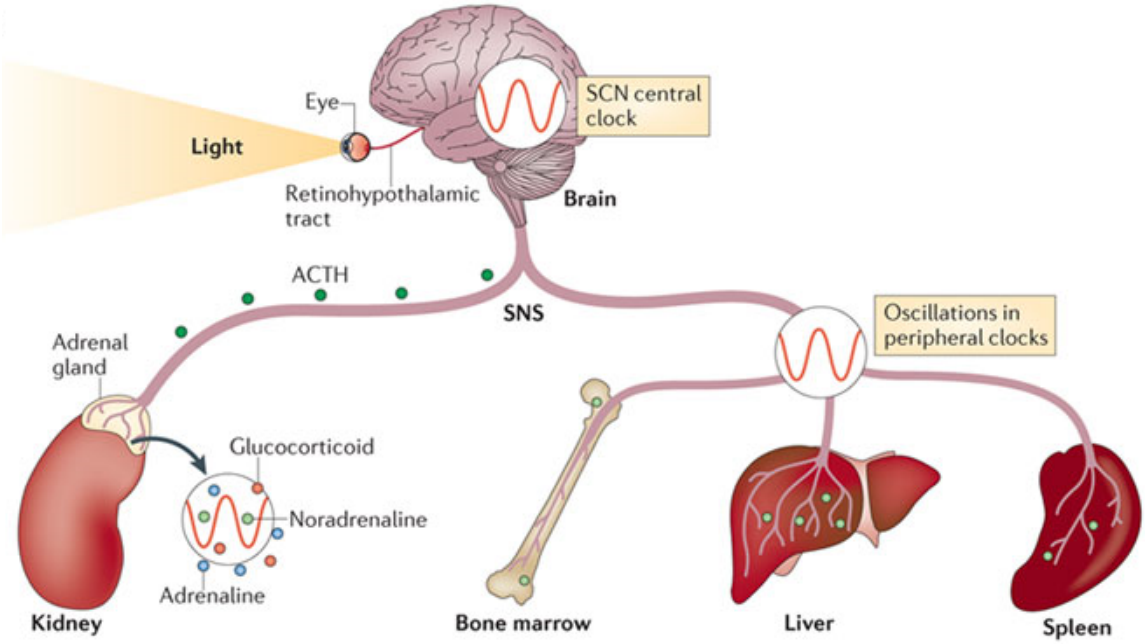

Figure 1: An overview of the central clock system.

The SCN generates global circadian rhythms, which are entrained to external cues (light). The synchronization of rhythms in the SCN leads to clock modulation in peripheral tissues of humoral and neural output systems via the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and the sympathetic nervous system (SNS) to regulate the rhythmic release of hormones (figure adapted from [22]).

1.3 Positive and Negative Feedback Loops

At the molecular level, the circadian clock in mammals consists of several interacting transcriptional/translational positive and negative feedback loops involving at least 14 core-clock genes [5]. These genes encode for members of several gene families: PER (Period, *Per1*, *Per2* and *Per3*), CRY (Cryptochrome, *Cry1* and *Cry2*), BMAL (Brain and muscle ARNT-like protein, *Bmal1 (ARNTL*) and *Bmal2* (*ARNTL2*)), CLOCK (Circadian locomotor output cycles kaput), NPAS2 (neuronal PAS domain-containing protein 2, in neuronal tissue), ROR (retinoic acid receptor-related orphan receptor, *Ror-α* (*RORA*),

Ror-β (*RORB*) and *Ror-γ* (*RORC*)) and REV-ERB (nuclear receptor, reverse strand of ERBA, *Rev-Erbα* (*NR1D1*) and Rev-Erbβ (*NR1D2*)). In the central loop and during the early hours of the day, the transcription of *BMAL1* and *CLOCK* (or its related gene *NPAS2*) results in the heterodimerization of the BMAL1-CLOCK complex in the cytoplasm. This heterodimerization triggers its nuclear translocation and initiates transcription by binding to specific DNA elements, E-boxes (5′-CACGTG-3′) and E′-boxes (5′-CACGTT-3′) in the promoters of clock-controlled genes (CCGs) [26,27]. In addition, BMAL1 and CLOCK activate the transcription of *Per (Per1 and Per2)* and *Cry (Cry1 and Cry2)* genes, which belong to the negative limb of the feedback loop. The synthesized PER and CRY proteins subsequently heterodimerize and migrate to the nucleus, thereby repressing additional BMAL1-CLOCK transcriptional activity. This establishes the groundwork for the cycle's reiteration from a baseline of reduced transcriptional activity approximately every 24 hours. To start in a new cycle, the degradation of the negative feedback loop proteins, PER and CRY, becomes essential.

The BMAL1-CLOCK heterodimer also activates a second feedback loop which acts in coordination with the loop mentioned above. This loop consists of the nuclear receptors ROR (α, β and γ) and REV-ERB (α and β), which through E-box activation by BMAL1-CLOCK translocate back into the nucleus and compete for the Retinoic acid-related Orphan receptor Response Element (RORE) binding sites within the promoter of *BMAL1*. Whereas RORs promote *BMAL1* expression, REV-ERBs act to suppress it. It was previously thought that these nuclear receptors formed an accessory feedback loop that stabilizes the circadian clock. However, recent studies have refined this statement and suggested a more prominent role of RORs and REVs capable of driving circadian rhythms [28]. This was demonstrated in inducible double knockout strategies that have allowed the deletion of Rev-Erbα and β in an adult animal. In these animals,

Nr1d1$^{-/-}$ *Nr1d2*$^{-/-}$ mice showed a complete loss of circadian rhythm, comparable to other clock-deficient strains (such as *Per1*$^{-/-}$*Per2*$^{-/-}$ and *Cry1*$^{-/-}$*Cry2*$^{-/-}$) [28]. **Figure 2** shows the dominant interacting partners of the molecular circadian clock in mammals.

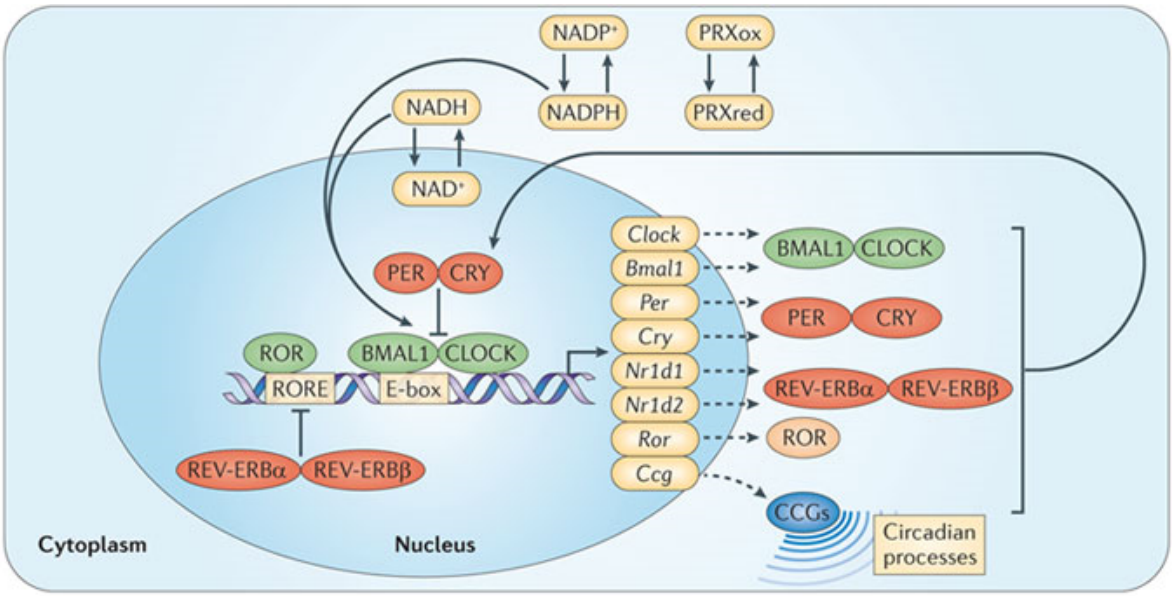

Figure 2: Positive and negative feedback loops of mammalian circadian clock.

A simplified overview of the major molecular components of the mammalian circadian clock. The heterodimerization of BMAL1-CLOCK activates the transcription of CCGs by binding to their E-box. This also includes the activation and heterodimerization of the PER-CRY complex, which in turn inhibits the transcription of BMAL1 and serves as the negative feedback loop. Additionally, nuclear receptors (RORs and REV-ERBs) are expressed in response to BMAL1-CLOCK activity. While RORs activate the expression of BMAL1, REV-ERBs repress it (figure adapted from [22]).

1.4 Peripheral Clocks

Nearly every mammalian tissue shows the presence of the transcriptional/translational feedback loops described above [29]. Neurons in the SCN coordinate a consistent temporal framework throughout the entire organism by aligning numerous peripheral clocks, which oscillate autonomously, thereby establishing a shared rhythm. These peripheral clocks consist of the same molecular components as in the SCN. In addition to the core-clock network (CCN) genes, there are hundreds or thousands of genes

expressed in a circadian manner. In fact, up to 80% of protein-coding genes exhibit circadian rhythms in their expression in at least one tissue in mammals [3]. Temporal synchronization within the organism is orchestrated indirectly through cyclic rest-activity patterns but also through a direct and intricate interaction, not yet fully elucidated, between key hormones (mostly glucocorticoids and catecholamines) and local autonomic neural networks [30,31]. Numerous factors contribute to the alignment of peripheral clocks, ensuring stability and enabling the organism to sustain its overarching rhythm despite disturbances in environmental cues (zeitgebers) or even compromised CCN functionality **(Figure 3)**. In a study on mice, 31 genes in the clock-less liver continued to oscillate even after a knockdown of the BMAL1-CLOCK expression in these cells, presumably by using the systemic signals from the rest of the animal [32]. The circadian regulators within these cells can be temporarily entrained through an array of signalling cascades, encompassing both transmembrane and intranuclear receptors [33]. Consequently, peripheral oscillators exhibit properties of self-sustainability, cellular autonomy, and robustness against cellular division as well as variations in temperature conditions and overall transcriptional activity.

Another strong zeitgeber signal in mammals is food. The SCN can modulate behavioural rhythms in rest–activity and food intake, which can indirectly synchronize peripheral clocks. Food intake can also independently synchronize peripheral clocks (most predominantly demonstrated in the liver), even in the absence of a functional circadian system or SCN [32,34]. It has been shown that, for example, time-restricted feeding can reset the phase of peripheral clocks, but not of the SCN in mice [35,36].

Also, exercise can phase-reset the circadian clock in muscle cells, possibly by inducing transient hypoxic stress and by activating *HIF1A*, which has been shown to bind to core-clock gene promoters and to build a heterodimer with BMAL1 [37,38].

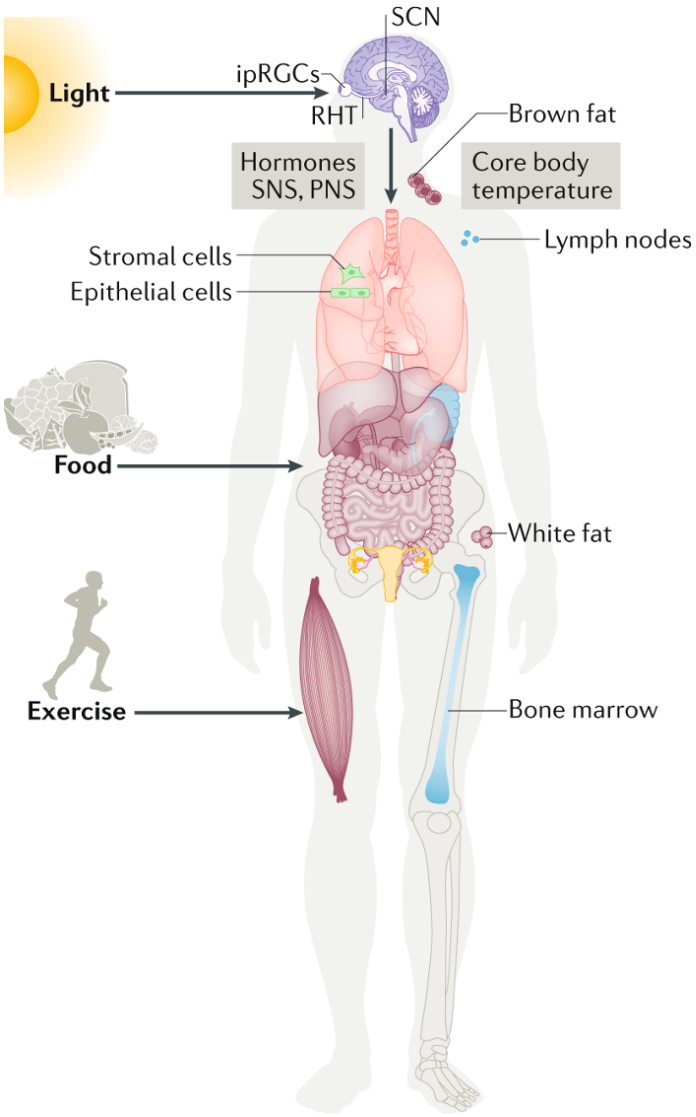

Figure 3: Synchronization of central and peripheral clocks in humans.

The SCN is entrained directly through light signals detected by intrinsically photosensitive retinal ganglion cells (ipRGCs) in the eyes and transmitted via the retinohypothalamic tract (RHT). The central clock (SCN) propagates synchronization cues to the entire body through hormones, the sympathetic and parasympathetic nervous systems (SNS and PNS, respectively), and oscillations in core body temperature. Additionally, the phase of the peripheral clocks (e.g., in liver and muscle cells) can be independently set according to certain environmental zeitgebers such as the timing of food intake and exercise (figure adapted from [39]).

1.5 Circadian Clock and Cancer

Oncogenic transformations lead to marked disruptions in cellular homeostasis, manifesting as abnormal proliferation and metabolic malfunctions [40]. Thus, malignant cells exhibit unprogrammed mitotic progression, leading to atypical and accelerated tissue proliferation. Concurrently, neoplastic cells exploit mechanisms to evade immune detection and resist programmed cell death, further facilitating malignant propagation and metastatic spread [40]. These distinct processes are integral to the well-recognized hallmarks of cancer [40], with emerging research elucidating their modulation via the circadian clock [41] **(Figure 4)**. For instance, numerous studies highlight a reciprocal interaction between the cell cycle and circadian rhythms in dividing cells (reviewed in [42]). Healthy cells predominantly adhere to a circadian-regulated division rhythm, with a rate of approximately 24 hours [43]. This periodicity is largely dictated by the interplay between cell cycle checkpoints and the intrinsic circadian molecular machinery [44]. Without external entrainment cues, the circadian clock consistently aligns to the cell cycle in a 1:1 ratio, manifesting with the *NR1D1* reporter expression reaching its peak around 5 hours post-mitosis [45]. As mentioned above, studies involving animal models and human tumour samples have elucidated that perturbations in circadian rhythms represent a significant intrinsic factor in the oncogenesis of mammalian cells. These disruptions can occur due to misalignments between the external and internal time of the organism, such as during shift work, or due to circadian alterations caused by mutations in single clock genes, such as *Per2* or *Bmal1*, which have resulted in accelerated tumour growth *in vivo* [46,47]. These outcomes are mainly because of an increase in proliferation rate upon circadian rhythm disruption, since tumour suppressor and key cell cycle genes are under clock control [11,44] in the context of a bidirectional clock-cell cycle coupling [45,48,49]. Furthermore, interactions between PER1 and key proteins integral to DNA damage response have

been identified and upregulation of *PER1* has shown inhibitory effects on the proliferation of certain human cancer cell lines [50]. Another CCG, the histone deacetylase sirtuin 1 (*SIRT1*) exhibits dual roles in tumorigenesis as it can either promote or suppress tumour growth, influenced by its interacting partners [51]. On the other hand, in cancer cells, several core-clock genes, including *CLOCK*, *BMAL1*, *PER2* and *NR1D1* are dysregulated and have been reported to play a role in tumorigenesis (reviewed in [52]). While *BMAL1*, *CLOCK*, *PER2* and *CRY2* were decreased in several human cancer samples including melanoma, pancreatic, colorectal, lymphoma, breast and thyroid cancer, *CRY1* expression was increased in colorectal cancer samples, pointing towards the differential effect of core-clock gene alterations in cancer progression [52].

Considering this, the past decade has seen the emergence of circadian medicine, premised on the existing asynchrony between cell proliferation and drug metabolism observed in normal versus malignant tissues. Notably, the deployment of cancer therapy anchored in circadian timing has reflected promising outcomes (reviewed in [41,53]), but lacks further investigations into the molecular interplay between the circadian clock and cellular growth regulation in order to enhance therapeutic efficacy [54,55]. As a general concept, enhancing circadian clock function has been shown to be a pivotal approach in providing both preventive and therapeutic strategies for various diseases, notably metabolic syndrome and cancer. This can be realized through precise behavioural modulations, chronologically optimized drug administration, and molecular strategies targeting specific circadian clock components (reviewed in [56]). Collectively, these findings indicate a reciprocal relationship between oncogenic transformation and disruptions in core-clock gene expression in human malignant cells.

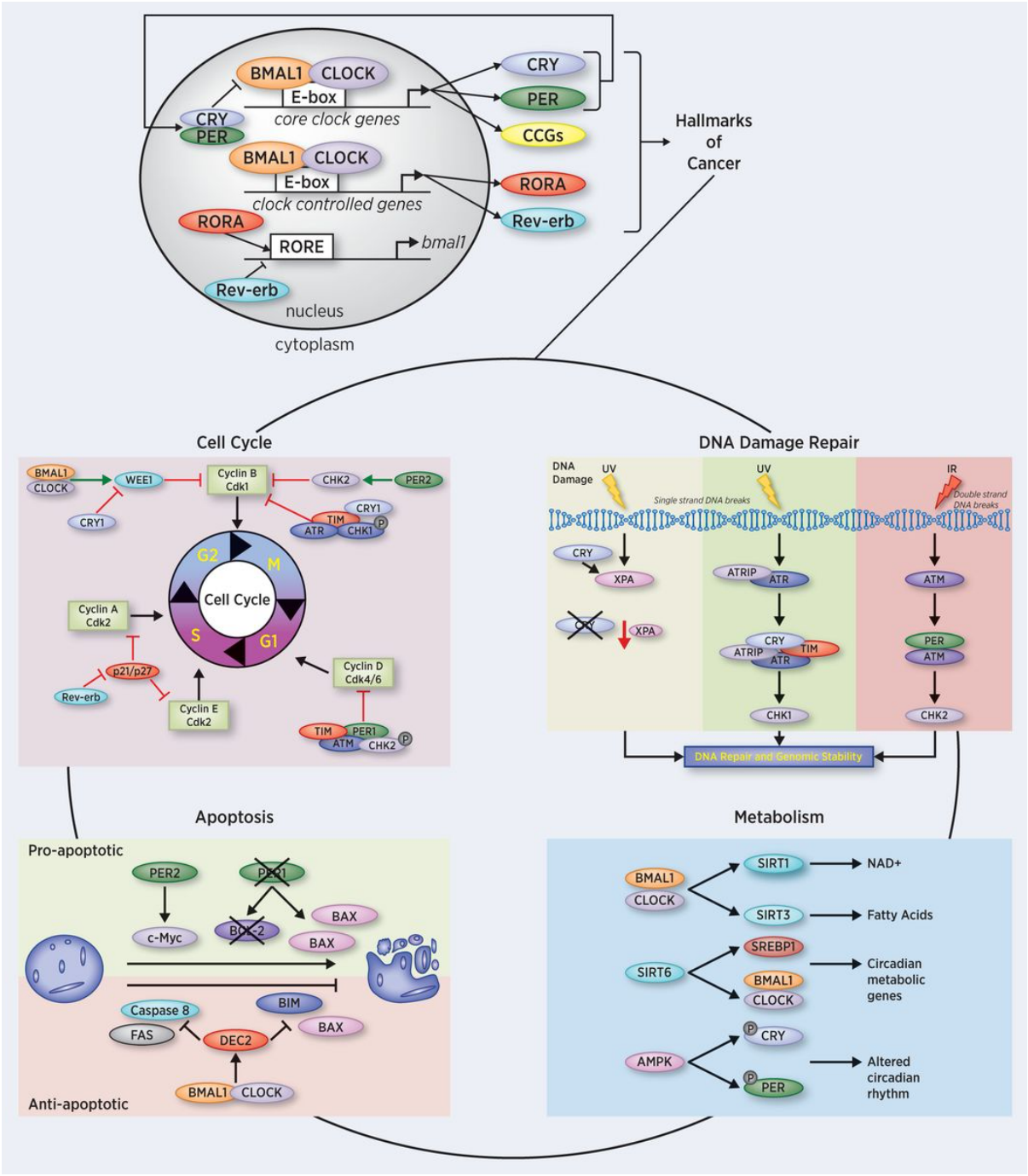

Figure 4: Disruptions of circadian rhythms affect several hallmarks of cancer.

The circadian clock is connected to several hallmarks of cancer, including the cell cycle, apoptosis, DNA repair, and metabolic regulation, which impact tumour development and progression. Therefore, the disruption of circadian rhythms by various factors may promote the formation of cancer hallmarks and play a fundamental role in tumorigenesis (figure adapted from [57]).

1.6 Project Aim

Given the mentioned body of experimental and clinical evidence linking the circadian clock to the genesis and progression of cancer and its potential implication in enhancing therapy in patients, the goal of my research is to gain a deeper understanding of the molecular interactions between the circadian clock and colorectal cancer (CRC) using *in vitro*, *in vivo*, and 3D cell modules, with a focus on cancer progression, invasion and metastasis **(Figure 5)**. To this end, I aim to tackle the following questions:

(1) How do perturbations in circadian core-clock elements affect cellular properties involved in CRC progression (e.g., proliferation, apoptosis and metastasis formation)?
(2) Which core-clock and/or cancer related element(s) might be part of this regulation?
(3) Can alterations in identified cancer related elements affect the circadian clock?

For this purpose, I used CRC cell lines from different origins with distinct circadian clock phenotypes (HCT116, SW480 and SW620) and performed perturbations on core-clock genes *BMAL1 (ARNTL)*, *PER2* or *NR1D1* and evaluated the effects on cancer processes related to epithelial-mesenchymal-transition (EMT) as an indication of cancer progression using *in vitro*, *in vivo* as well as 3D culture assays (chapter 1). I found that clock perturbation affected cell motility and invasiveness in CRC cells and identified a promising EMT-related element affecting both cancer metastasis potential and circadian clock function, possibly serving as an interacting partner linking the two systems (chapter 2).

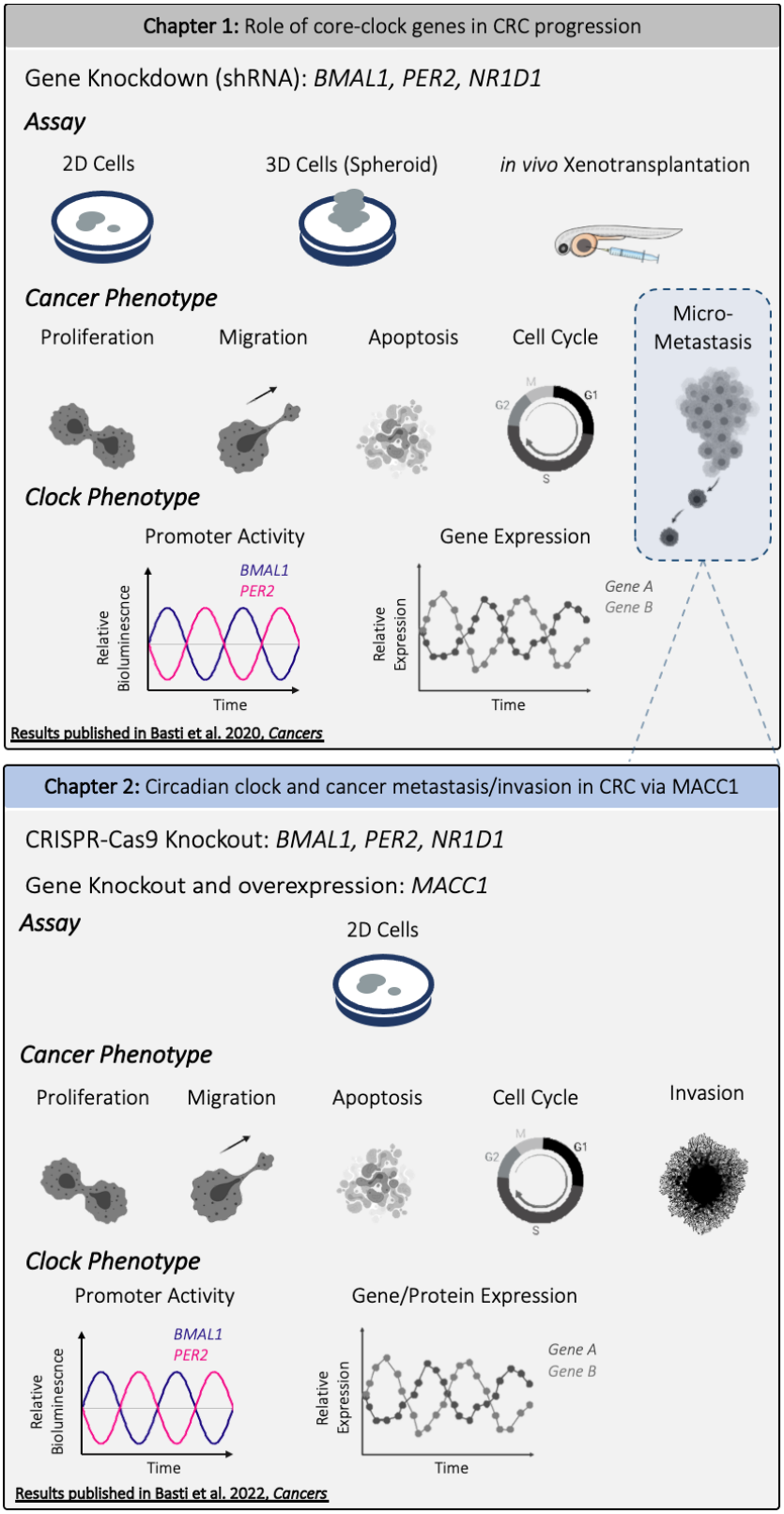

Figure 5: Overview of the experimental approach.

This schematic illustrates the experimental strategy used to address the objectives outlined in the project aim section of the thesis. Icons were partly created with BioRender.com

2 Summarized and Selected Methods

2.1 Cell Lines

We used CRC cell lines of different progression stages and origins to investigate the putative role of core-clock genes in regulating cancer progression and metastasis. The cell lines used were HCT116 and SW480, which are derived from primary tumours, and SW620, which is the metastatic counterpart of SW480. Bioluminescence live-cell measurements were used to monitor the circadian oscillations of the cells. HCT116 cells displayed a robust circadian oscillation, while SW480 and SW620 cells had a moderate and weak oscillation pattern, respectively [6,13].

To assess the impact of circadian clock perturbation in these cells in chapter 1, we generated cell lines with stable knockdown (KD) of core-clock genes *BMAL1*, *PER2* or *NR1D1* using shRNA lentivirus and analysed both circadian clock and cancer cell properties. In chapter 2 and in addition to KD cell lines, we generated core-clock manipulated CRC cells by knocking out (KO) *ARNTL (BMAL1)*, *PER2*, or *NR1D1*. The CRISPR-Cas9 system was used to generate core-clock knockout cells in HCT116. All KO and KD cells were compared with respective control cells and investigated for clock (*BMAL1* promoter activity) and cancer cell (proliferation, apoptosis, and invasion) characteristics. To determine the role of *MACC1* in CRC progression and its interplay with the circadian clock, *MACC1* was manipulated by overexpression or KO in CRC cells. *MACC1* overexpressing and knockout cells were also compared with control cells for circadian clock and cancer phenotype characteristics.

2.2 Live-cell Bioluminescence

We measured live-cell bioluminescence recordings of *BMAL1* or *PER2* promoter activity in synchronised cells as indicators of circadian phenotype in manipulated and control cells and compared circadian parameters (amplitude, period and phase)

between the different conditions to the control condition. For this, all cell lines were first stably transduced with lentiviral elements containing either a *Bmal1*-promoter-driven luciferase or a *Per2*-promoter-driven luciferase as previously described [58]. The cells were synchronized through a medium change. The activities of the *Bmal1*-promoter-reporter or *Per2*-promoter-reporter were measured over five days using a LumiCycle instrument. The raw data were de-trended using the 24-hour running average with the Chronostar analysis software V3.0 [59]. The initial 12 hours of measurement, which were particularly noisy due to the instrument's limitations, were excluded from the analysis. Finally, the phase in radian was calculated using the following formula:

$$\varphi(rad) = \varphi(h) \times 2\pi/T$$

with $\varphi(h)$ = phase (in h), T = period

2.3 *In vitro* Live-cell Analyses

For cancer cell related effects, we measured cell proliferation, apoptosis and migration *in vitro* using a live-cell analysis system (IncuCyte) for several days (chapter 1). This allowed us to gain a deeper insight into altered cellular dynamics upon circadian clock dysregulation at a higher temporal resolution with minimal cell perturbation. Additionally, to determine cell invasion potential upon core-clock or *MACC1* manipulation in CRC cells (chapter 2), we performed chemotaxis cell invasion assay over time using live-cell imaging, which evaluates 3D chemotactic cell invasion through a biomatrix, as an indication of cancer invasiveness and metastatic potential. 3D cell invasion was quantified by measuring total phase object area on the bottom layer of the inner chamber normalized to the initial phase object area of the top layer.

2.4 *In vivo* Analyses

For the *in vivo* assessment of circadian clock disruption on tumorigenesis, we injected HCT116 cells with stable KD of *BMAL1*, *PER2* or *NR1D1* into 2 days post-fertilisation zebrafish embryos (chapter 1). At 4 days post-injection (4 dpi), we assessed the consequences of clock gene downregulation on the previously investigated cancer hallmarks: cellular proliferation (through mitotic figure quantification), apoptosis (by measuring activated caspase 3 levels), tumour size, and metastatic potential. To evaluate metastatic potential, CRC cells were injected either in the Perivitelline Space (PVS) only, or directly into circulation (similar to experimental metastasis in mouse). In the initial approach (using only PVS), to effectively form a micrometastasis, tumour cells must navigate through all stages of metastatic formation, from early to late steps. This includes invading adjacent tissues, entering blood vessels through intravasation, enduring in the circulatory system, and subsequently extravasating to colonise a remote location. However, using the second method, cells only need to survive circulation, extravasate and colonise, which represents later metastatic steps. Therefore, using this approach we could evaluate the metastatic potential and the ability of cells to undergo different behaviours to perform metastasis.

2.5 Cell Cycle

We complemented our *in vitro* data with measurements of cell cycle phase distribution in synchronized cells, to gain a deeper understanding of cell cycle dynamics upon circadian clock and/or *MACC1* manipulation and to see whether a) alterations in cell cycle dynamics correlate with changes in cell apoptosis and proliferation (chapter 1) and b) core-clock or *MACC1* alterations affect cell cycle in a similar manner (chapter 2). For this, we quantified cell cycle phase distributions using flow cytometry in synchronized fixed cells with a DNA staining dye (propidium iodide).

2.6 Time-course Gene and Protein Expression

To evaluate whether alterations in core-cock or clock-related elements affect oscillations in gene or protein expression we performed time-course experiments of key genes involved in the regulation of cell cycle checkpoints (*MYC* and *WEE1*) and in cell apoptosis (*TP53*) in circadian clock dysregulated cells (chapter 1) as well as MACC1 protein expression in core-clock KO cell lines (chapter 2). To do so, we synchronized cells using medium change and collected RNA or protein every three hours for 30 hours. With this, we aimed to understand whether changes in cellular dynamics (e.g., proliferation and apoptosis) are linked to changes in the mean or rhythmic expression of genes involved in cell cycle or DNA damage response, assumed to be under circadian control, and to examine whether MACC1 is under circadian control in CRC. In addition, we evaluated whether core-clock elements interact with MACC1 via protein-protein interactions using co-immunoprecipitation assay (chapter 2).

3 Main Results

3.1 Chapter 1: The Core-Clock Gene *NR1D1* Impacts Cell Motility *In Vitro* and Invasiveness in a Zebrafish Xenograft Colon Cancer Model

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Own contributions: all *in vitro* experiments, data and statistical analysis of *in vitro* experiments, visualisations, major parts of the text of the publication

3.1.1 Context

Circadian core-clock elements play a role in affecting cellular properties of colorectal cancer: As mentioned above, mounting evidence points to the regulation of several cancer hallmarks via the circadian clock [41] and thus, malfunctions of the biological clock system are associated with cancer progression and play a role in cancer therapy [61]. Examples of cancer-related genes regulated by the circadian clock machinery are *MDM2* and *VEGF*, involved in proliferation and angiogenesis [62,63], the glycolytic enzyme pyruvate kinase [64] and *HKDC1* involved in metabolism [13], transforming growth factor β (TGFβ) transduction pathway and *SMAD4* regulating oncogenic transformation and tissue homeostasis [65] as well as cell cycle checkpoint regulators *WEE1*, *MYC*, *CDKN2* and *cyclin D1* [5,14,66]. Another cell cycle element that interacts with the circadian clock is TP53, which serves both as a key element in mediating DNA damage response and cell cycle. TP53 interacts with a

specific response element in the *PER2* promoter, overlapping with an E-box, subsequently attenuating the transcriptional activity driven by CLOCK/BMAL1 [67].

As of 2020, colorectal cancer (CRC) ranks as the third most commonly diagnosed (10.0%) and the second most deadliest cancer (9.4%) worldwide (compared to all new cancer cases and cancer deaths, respectively) [68]. In CRC, the expression of core-clock genes is altered in both patient samples [69] as well as cancer cell lines [6,70]. In human CRC cells, *ARNTL*, *PER1* and *CRY2* appear to exert oncogenic properties [50,70,71], whereas *CRY1* exhibits tumour-suppressive activities upon downregulation [72]. These data elucidate the multifaceted regulatory interplay of the circadian clock in CRC, characterized by distinct outcomes upon modulation of core-clock gene expression. In addition, clinical trials utilizing chrono-modulated treatment in patients with metastatic CRC show promising results [73-75] and the use of circadian clock as biomarker for evaluating CRC patient survival and monitoring patient response [76-78] makes this a suitable and very interesting model system to study clock-cancer interactions. However, the underlying mechanisms of the circadian control in CRC, particularly regarding cell invasiveness and metastasis potential is not fully unravelled and require further investigations.

Zebrafish as an emerging *in vivo* model for cancer studies: In recent years, zebrafish larvae xenografts have become an exciting and suitable model organism for *in vivo* and single cell cancer studies [79], especially with a focus on cancer invasion potential and tumour microenvironment [80]. Compared to other *in vivo* model organisms (e.g., chick embryo and mouse), zebrafish larvae offer accessible single cell analysis, which makes it possible to quantify the impact of molecular alterations of circadian clock components in tumorigenesis (e.g., tumour size and apoptosis). Given

that the adaptive immune response in zebrafish embryos is not fully operational until 9–12 days post-fertilization, this model offers a suitable environment for (xeno)-transplantation studies. Furthermore, the zebrafish embryo model offers the power of replicates and ease of handling, which is advantageous compared to other similar model organisms.

3.1.2 Findings

Knockdown of clock genes affects oscillatory phenotype of colorectal cancer cells *in vitro*: Interestingly, we found that the KD of any of the core-clock genes *BMAL1*, *PER2* or *NR1D1* altered both clock and cancer cell properties in CRC cells, though with gene-specific manifestations (**Figure 6**). When looking at circadian clock properties, we observed a complete loss of circadian rhythms after *BMAL1* KD, pointing to its central role in maintaining proper circadian oscillations in cells, while *PER2* KD or *NR1D1* KD led to more subtle changes in amplitude and/or period of oscillations (**Figure 6A**). We observed a significant decrease in the period of oscillations for sh*NR1D1* cells ($p < 0.01$) and a phase advance for the sh*PER2* cell line ($p < 0.05$) (**Figure 6B**). Transcriptional analysis revealed significant and differential modulations in core-clock gene expression, specifically for *PER2*, *CRY1*, *NR1D1*, *CLOCK*, and *BMAL1*, across the distinct KD cell lines relative to the control line, 24h post-cell synchronization (**Figure 6C**). Overall, the data highlight the integral contribution of the core-clock machinery in orchestrating the oscillatory profile of HCT116 cells. This, in turn, potentially influences the transcriptional activity and subsequent cellular functionality of downstream CCGs.

Downregulation of core-clock genes affects cellular functioning *in vitro*: Next, we investigated the potential effect of perturbing core-clock genes in cellular functioning in CRC cells by measuring their proliferation and apoptotic rates compared to control cells. We found that all KD cell lines exhibited higher proliferation rates compared to the control, highlighting a putative role for the biological clock as tumour suppressor [13,70,81] (**Figure 6D**). Our cell cycle analysis showed significant changes in the distribution of cell cycle phases for both sh*BMAL1* and sh*PER2* cell lines compared to the control, with a decreased G0/G1 phase after *BMAL1* KD ($p < 0.05$) and increased S and G2 phase in sh*PER2* cell line ($p < 0.001$) (**Figure 6E**).

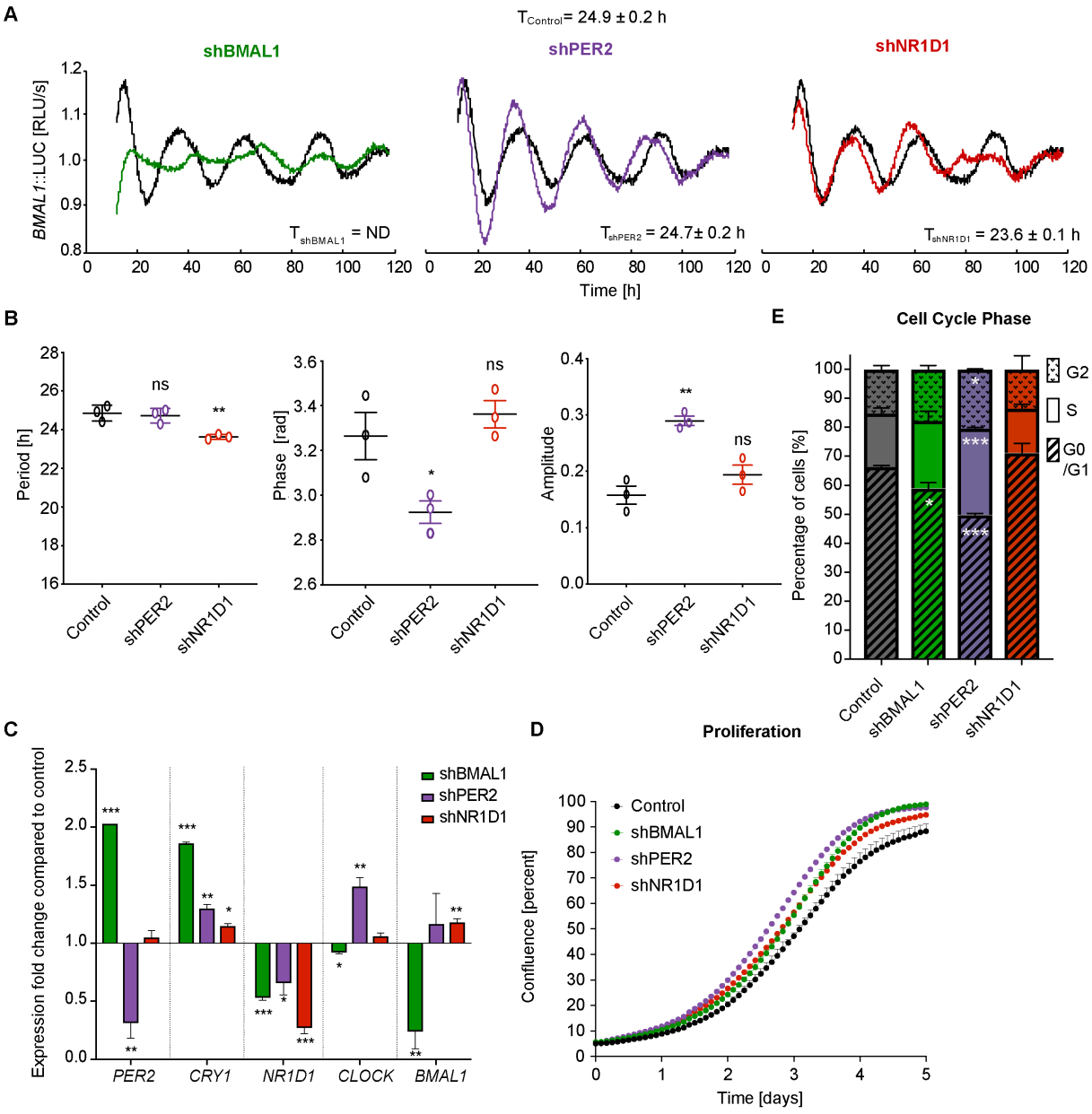

Figure 6: Knockdown of core-clock genes affects circadian clock and cellular functions in CRC cells.

(A) Bioluminescence readouts for the promoter activity of *BMAL1* over the course of 120h in HCT116 control and knockdown (sh*BMAL1*, sh*PER2* and sh*NR1D1*) cell lines (n = 3, mean ± SEM). (B) Period, phase and amplitude analysis of circadian bioluminescence data using Chronostar software [59]. (C) Gene expression analysis of core-clock genes *PER2, CRY1, NR1D1, CLOCK* and *BMAL1* in HCT116 control and shRNA knockdown cell lines at 24h after synchronization (n = 3, mean ± SEM). ND, not defined. ns or no asterisk $p > 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed unpaired t-test. (D) Proliferation analysis of HCT116 control and shRNA knockdown cell lines over 5 days (n > 8, mean ± SEM, $p < 0.001$ for sh*BMAL1*, sh*PER2* and sh*NR1D1* comparing AUC to control, two-tailed unpaired t-test). (E) Cell cycle phase distribution of KD cell lines compared to control (n = 3, mean ± SEM, no asterisk p > 0.05, * p < 0.05, *** p < 0.001; two-tailed unpaired t-test). Figure adapted from [60].

To better understand the underlying cellular changes, we conducted a time-course gene expression analysis on key genes responsible for regulating cell cycle checkpoints (*MYC* and *WEE1*) and cell apoptosis (*TP53*) in synchronized HCT116 KD and control cells (**Figure 7A**). We found that *MYC* and *WEE1* exhibited significant circadian oscillations in the control cells ($p < 0.05$), but not in any of the KD cells, indicating a circadian disruption of gene expression. However, we detected significant oscillation of *TP53* in sh*BMAL1* cells ($p < 0.05$), indicating a differential regulation of *TP53* via *BMAL1*. Indeed, the average expression of the mentioned cell cycle and apoptosis-related genes were significantly upregulated in sh*BMAL1* cell line ($p < 0.001$) (**Figure 7B**). The aberrant expression of *TP53* in sh*BMAL1* cells, coupled with its differential circadian modulation, could potentially impact cell apoptosis. To test this, we measured caspase 3/7 fluorescent activity over several days using live-cell imaging and found that sh*BMAL1* cells showed significantly higher apoptosis compared to the control, while apoptotic rates were significantly decreased in sh*NR1D1* cells ($p < 0.05$) (**Figure 7C**). These data also showed that apoptosis rates exhibited a dependency on the distinct downregulation of core-clock genes, which were in line with previous studies assessing the impact of *BMAL1* overexpression and *NR1D1* activation on

HCT116 cells, manifesting as reduced cell proliferation and elevated apoptosis induction, respectively [70,82]. When investigating the migratory behaviour of CRC cells *in vitro* using a wound-healing assay, we showed that downregulation of *BMAL1* or *NR1D1* led to a significant decrease in CRC motility ($p < 0.05$), suggesting that the circadian clock genes play a role in cell motility (**Figure 7D**).

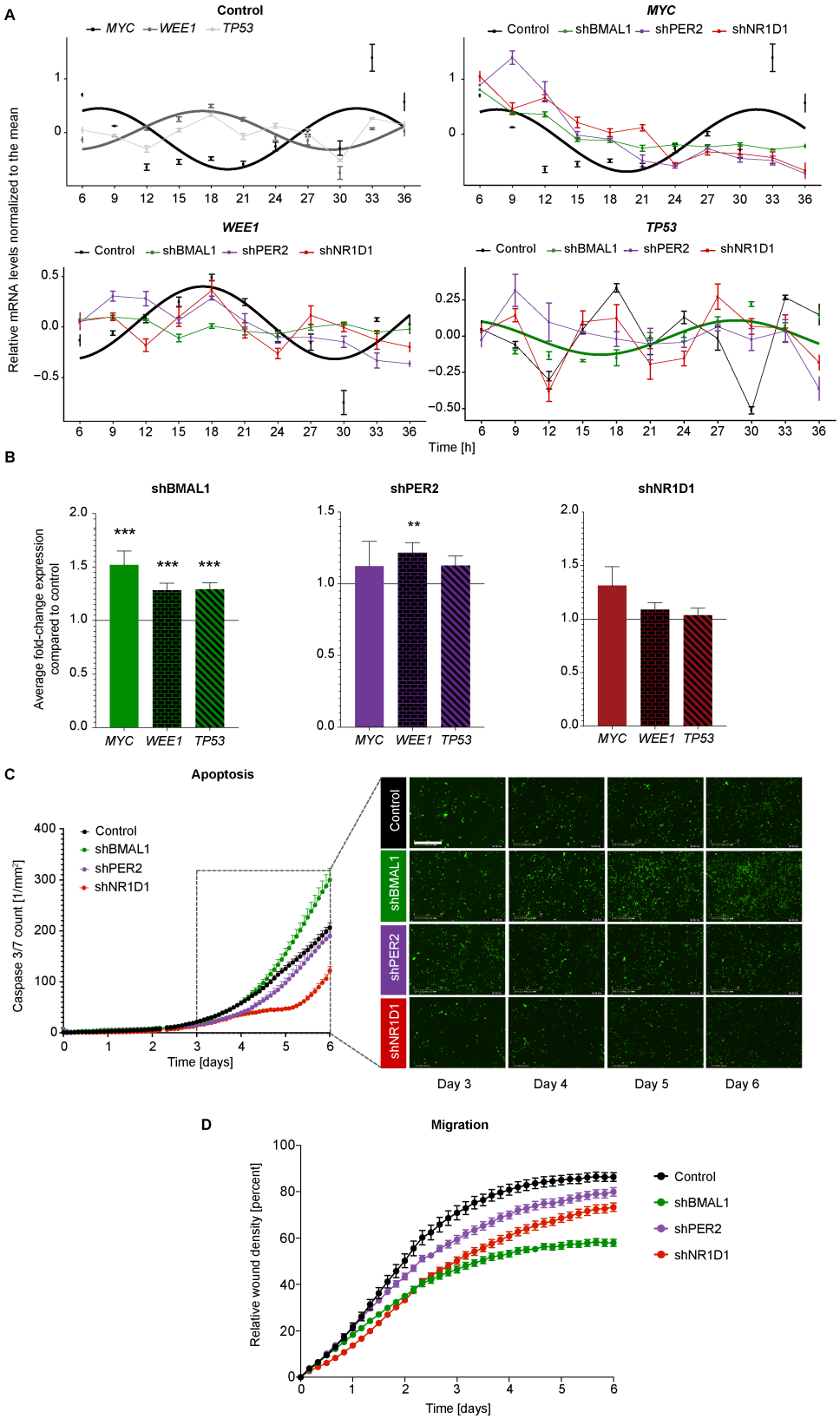

Figure 7: Alteration of core-clock genes affects the circadian regulation of cell cycle and apoptosis related genes and impairs cell motility *in vitro*.

(A) 30-hour time-course gene expression analysis for *MYC*, *WEE1* and *TP53* in control and different HCT116 KD cells (n = 3, mean ± SEM, a cosine curve was fitted to all data sets and displayed as a full line for $p < 0.05$, the data points were connected with closed lines if $p > 0.05$). (B) Average expression level of *MYC*, *WEE1* and *TP53* in KD cell lines compared to the control (n = 3, mean ± SEM, no asterisk $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed unpaired t-test). (C) Apoptosis analysis of HCT116 KD cell lines using caspase 3/7 marker (n > 8, mean ± SEM, $p < 0.05$ for sh*BMAL1* and sh*NR1D1*, two-way ANOVA and corrected for multiple testing using Benjamini and Yekutieli method. Measurements obtained by counting caspase 3/7 green objects per mm^2 every 2h over 6 days using the IncuCyte S3 device. Representation of the apoptosis assay using the IncuCyte S3 software over 6 days. Green dots represent apoptotic cells expressing caspase 3/7. Scale bar: 500 µm. (D) Cell migration analysis of control and shRNA KD HCT116 cell lines (sh*BMAL1*, sh*PER2* and sh*NR1D1*). Measurements were obtained using a scratch wound assay (IncuCyte). Quantification was performed by measuring the relative wound density over 6 days (n > 5, mean ± SEM, $p < 0.05$ for sh*BMAL1*, sh*PER2* and sh*NR1D1* compared to control, two-way ANOVA and corrected for multiple testing using Benjamini and Yekutieli method). Figure adapted from [60].

Downregulation of *BMAL1* or *NR1D1* affects tumorigenesis *in vivo*: We used a zebrafish xenograft model to test the impact of downregulating core-clock genes (*BMAL1*, *PER2*, and *NR1D1*) on tumorigenesis (proliferation, apoptosis, tumour size, and metastatic potential) (**Figure 8A-F**). Our data showed that downregulation of *BMAL1* led to a significant reduction in proliferation and tumour size ($p < 0.001$), as well as an induction of apoptosis ($p < 0.05$) but did not have a significant impact on the metastatic potential. However, downregulation of *NR1D1* led to a significant reduction in the capacity of cells to form micrometastasis in the tail region by 75% when injected directly into circulation ($p < 0.001$). Interestingly, we observed an overall decrease in cell proliferation and tumour size in the zebrafish xenograft model, which contrasted with the *in vitro* results where downregulation of core-clock genes resulted in increased cell proliferation. To investigate the potential influence of the zebrafish host environment on the circadian clock of HCT116 xenotransplants in comparison to their *in vitro* state, we introduced HCT116 cells into zebrafish embryos. Subsequently, we collected xenograft-bearing zebrafish larvae at 3-hour intervals over a 24-hour period

under dark/dark conditions, thereby eliminating potential light-induced perturbations on the zebrafish circadian clock (**Figure 8G**). We found that the temporal as well as the average expression level of the human *PER2* gene in the xenografts were dramatically altered compared to cells *in vitro*, indicating that CRC cells inside the xenografts had a different circadian clock than the cells outside of the zebrafish (**Figure 8H-I**).

Taken together, our data illuminate the pivotal roles of *BMAL1* and *NR1D1* in the viability of HCT116 tumours and highlight a distinct mechanistic role for *NR1D1* in influencing metastatic potential in an *in vivo* setting.

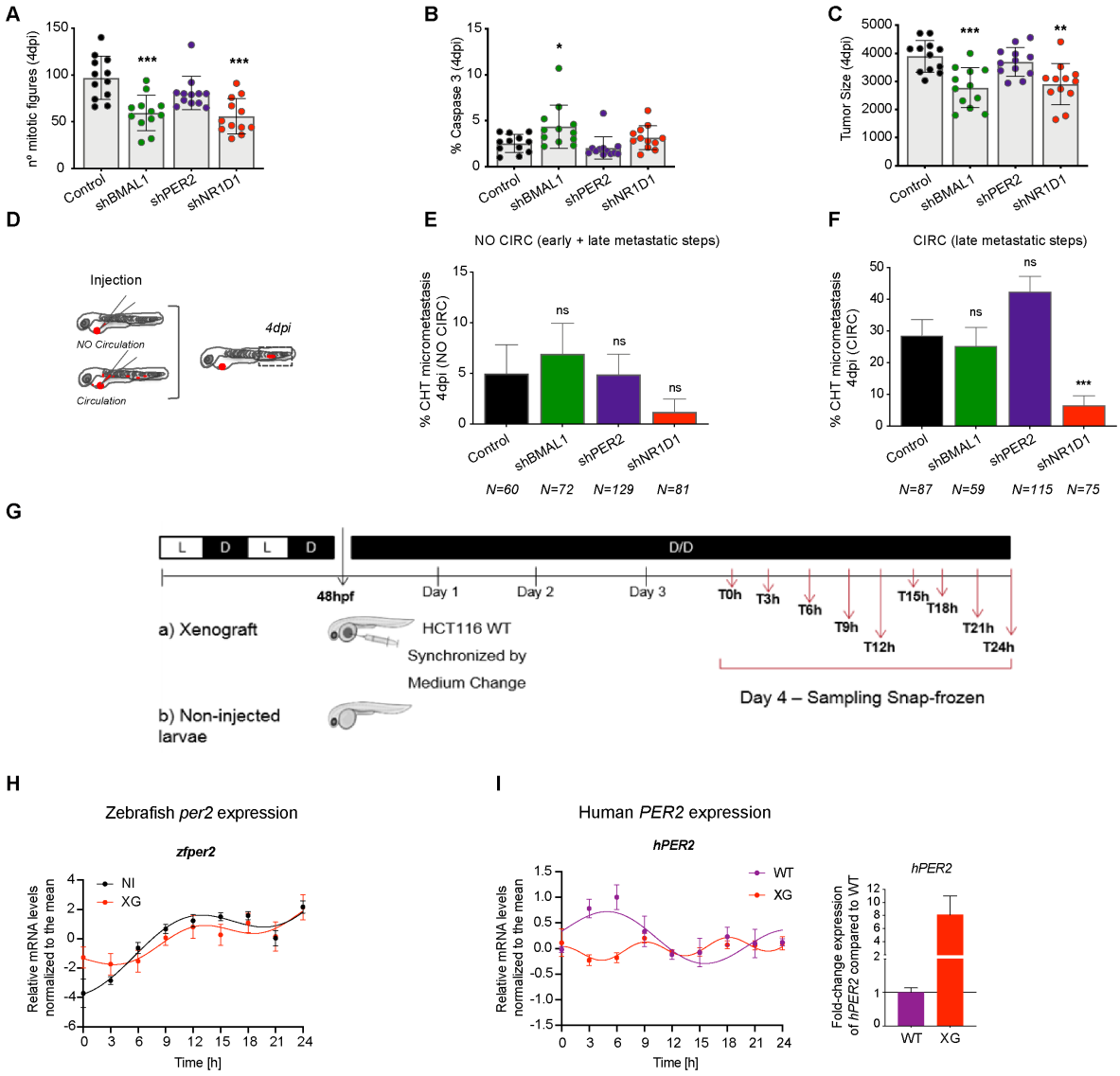

Figure 8: Downregulation of core-clock genes affects tumorigenesis *in vivo*.

At 4 days post-injection (dpi): (A) mitotic figures, (B) % of apoptosis, (F) activated caspase 3, (C) tumour size (number of tumour cells) and (D-F) metastatic potential were quantified. Metastatic potential was quantified by injecting cells into the Perivitelline Space (PVS) only, with no cells in circulation (E) and cells injected directly into circulation (F). Each dot represents one xenograft. Results are from one single independent experiment. Statistical analysis was performed using Mann–Whitney test (ns or no asterisk $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Error bars: mean ± SEM. (G) Overview of the time-course assay for the *in vivo* study. HCT116 cells were injected into zebrafish larvae at 48h post-fertilisation (48hpf). After 3 days, xenograft larvae (XG) were collected every 3h for the course of 24h during dark/dark conditions. Non-injected zebrafish larvae were used as controls (NI), n = 45 larvae/time-point. Time-course mRNA expression levels compared to the mean for: (H) zebrafish *per2* gene in non-injected zebrafish larvae (black line and dots) and zebrafish xenografts injected with HCT116 cells (red line and dots), (I) human *PER2* gene in zebrafish xenografts injected with HCT116 cells (red line and dots) and in HCT116 WT cells *in vitro* (WT, purple line and dots). The average *hPER2* expression between XG and HCT116 WT cells is depicted as a bar plot. Cosinor fitting curves were applied for the determination of oscillation parameters (no asterisk $p > 0.05$). Figure adapted from [60].

3.1.3 Discussion

In this work, we demonstrated that the core-clock gene *NR1D1* plays an important role in regulating cell proliferation, motility and invasion in colon cancer cells. *NR1D1* is involved in the regulation of various cellular processes, including metabolism, proliferation, and differentiation [82]. Several studies have shown that *NR1D1* expression is frequently downregulated in various types of cancer, including breast, prostate, and liver cancers [83,84]. This reduced expression of *NR1D1* has been associated with a more aggressive phenotype and poorer prognosis in these cancers.

However, this study is the first to identify a role for *NR1D1* in regulating cell motility and invasion in cancer cells. The ability of cells to migrate and undergo several steps to metastasize is an important hallmark of cancer with profound therapeutic potential. Both our *in vitro* and *in vivo* data indicate a diminished cellular motility and

approximately a 20% reduction in metastatic potential following *NR1D1* suppression in HCT116 cells.

We speculate that *NR1D1* may regulate the expression of genes involved in cell-cell interactions, cell adhesion and migration as well as EMT, which could impact cell shape and motility. EMT is a process in which epithelial cells transform and acquire characteristics of mesenchymal cells. This transformation enhances cell migration, invasion, and contributes to drug resistance and is a crucial step in the development of metastatic CRC [85]. To check for this, we measured the expression of genes involved in EMT and CRC stem cell marker in downregulated *NR1D1* cells and observed a significant reduction of *SNAI1 (*$p < 0.001$*)* and *CD44* ($p < 0.01$) compared to the control cells (data shown in published article).

As a therapeutic approach, targeting NR1D1 has shown promising results in cancer studies. For example, in breast and gastric cancer cells, *NR1D1* showed anti-proliferative effects, either by targeting cyclin A2 to pause the cell cycle [86] or by inhibiting glycolytic flux and the pentose phosphate pathway [87], respectively. Also, pharmacological targeting of NR1D1 using agonists such as SR9009 and SR9011 has been suggested as a promising strategy for cancer treatment, as these drugs induce cancer cell death without affecting non-transformed cells [82]. Therefore, targeting NR1D1 could represent a novel therapeutic strategy for inhibiting tumour metastasis, in addition to other mentioned effects.

We furthermore suggest that the zebrafish xenograft model used in this study could be a valuable tool for future cancer research, as it allows for the visualization of cancer cell growth and invasion in real-time, using multiple biological replicates.

Finally, we acknowledge limitations of our work, including the fact that the zebrafish xenograft model may not fully recapitulate the complex microenvironment of human tumours. While this model allows for visualization and quantification of cancer cell invasion and metastasis, further studies in animal models or human patient samples are needed to confirm the relevance of these findings to human cancer. Also, further studies are needed to confirm whether *NR1D1* plays a similar role in regulating cell motility and invasiveness in other types of cancer cells.

In summary, this study suggests that core-clock genes play a role in regulating cell migration, invasion, and metastatic potential in colorectal cancer cell lines and proposes that the *NR1D1* may regulate these properties.

3.2 Chapter 2: Core-Clock Genes Regulate Proliferation and Invasion via a Reciprocal Interplay with MACC1 in Colorectal Cancer Cells

Published at the journal *Cancers* on the 16th of July 2022. The publication [88] with DOI: https://doi.org/10.3390/cancers14143458 is available at: https://www.mdpi.com/2072-6694/14/14/3458.

Own contributions: all *in vitro* experiments, data and statistical analysis of *in vitro* experiments, visualisations, major parts of the text of the publication

3.2.1 Context

As mentioned above and discussed in chapter 1 [60], circadian rhythms are regulated by a set of core-clock genes that play a critical role in maintaining the timing of physiological processes in various tissues and organs. Disruption of the circadian clock has been associated with several pathological conditions, including cancer, and circadian disruption is common in cancer cells and tumours, including colorectal cancer (CRC).

Circadian clock and colorectal cancer metastasis: importance and implications

As CRC metastasis is a significant cause of cancer-related morbidity and mortality worldwide, understanding the molecular mechanisms underlying CRC development and progression is crucial for providing effective treatment strategies. Circadian-based therapy has demonstrated a prominent role in CRC patients with increased efficacy and survival rates compared with conventional therapy [74,75]. Actigraphy measurements and wrist-based accelerometric analyses have also been used for monitoring and predicting therapeutic responses as well as evaluating clinical outcome

in patients with colorectal cancer [77,89]. Therefore, understanding the bidirectional connection between circadian clock components and cancer invasion and metastasis on a molecular basis opens new possibilities for treatment development and optimization.

In our previous work, we identified potential regulatory roles of *ARNTL* (also known as *BMAL1*) and *NR1D1* in modulating oncogenic parameters including cellular proliferation, apoptosis, and metastatic potential [60]. This generated a hypothesis suggesting that circadian clock components may regulate genes integral to metastatic processes via pathways including EMT, cell cycle dynamics and cellular invasion. Therefore, in our second study, we primarily focused on *MACC1* (metastasis-associated in colon cancer 1), which is well-known to be a driver and facilitator of cancer metastasis, particularly in CRC [90,91], yet its interplay with the circadian clock remains unexplored. MACC1 is a transcription factor involved in EMT, regulates expression of metastasis-related genes such as *c-MET* (which can directly induce metastasis), impacts tumour cell migration and invasion, and induces metastasis in solid cancers [90].

We investigated the extent of circadian clock control in CRC cell progression (e.g., proliferation and migration) via core-clock and clock-controlled genes, which regulate cell migration and invasiveness and provided evidence of a reciprocal interplay between *MACC1* and circadian clock, with an impact on CRC progression, particularly cancer cell proliferation and invasion.

3.2.2 Findings

EMT-related gene expression is affected by core-clock manipulation: Our first interesting observation was that the KO of core-clock genes *ARNTL*, *PER2* or *NR1D1* resulted in differential expression of several genes involved in EMT- and metastasis-related pathways, including *ECAD*, *SNAI1*, *CD44* and *CD133 (***Figure 9A***)*. Strikingly, *MACC1* showed the strongest differential expression, with a more than 3-fold increase after *ARNTL* KO and *PER2* KO, and a slight downregulation after *NR1D1* KO in HCT116 cells. Also, upon stable downregulation of the mentioned core-clock genes, we noted significant differential expression of *MACC1* in both SW480 and SW620 cell lines (**Figure 9B-C**). Thus, our gene expression data, together with observed changes in cell proliferation, apoptosis and migration upon core-clock gene manipulation (**Figure 9D**), suggest that disrupting the circadian clock machinery may play a role in promoting cancer-associated properties in CRC cells, particularly in EMT and cell invasion related pathways.

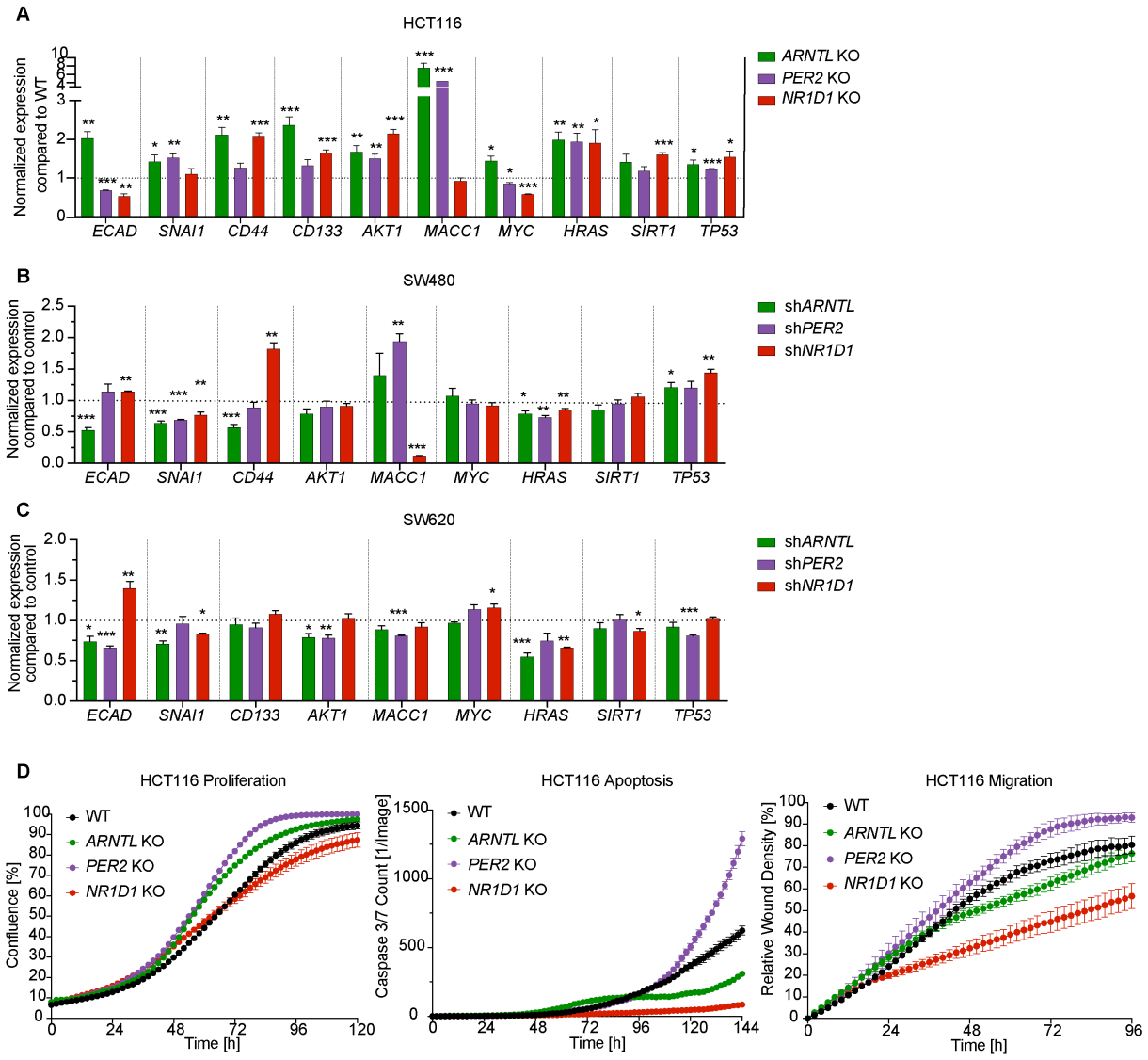

Figure 9: Core-clock manipulation alters EMT-related gene expression in CRC cells and affects cancer cell properties.

Gene expression analysis of related genes in EMT, cell cycle, death and metastasis in (A) HCT116, (B) SW480 and (C) SW620 WT/control and core-clock manipulated cell lines at 24h after synchronization (n = 3, mean ± SEM). (D) Proliferation, Apoptosis and Migration analysis of HCT116 WT and core-clock knockouts using live-cell imaging over several days (n > 8, mean ± SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed unpaired t-test. Figure adapted from [88].

The cellular circadian clock and cancer properties are both impacted by *MACC1* manipulation*:* Since *MACC1* showed the most striking expression change upon the KO of core-clock genes, we analysed a cellular model of *MACC1* overexpression and KO cells in HCT116, as well as *MACC1* manipulated SW480 (overexpressed) and SW620 (knocked out) cells. With this, we aimed to examine whether *MACC1* affects the circadian clock machinery. Indeed, gene expression analysis suggested a putative correlation between *ARNTL*, *NR1D1*, and *MACC1* expression (**Figure 10A-B**). The deletion of *MACC1* in HCT116 reduced the expression of *NR1D1* and *ARNTL* significantly ($p < 0.001$), while overexpressing *MACC1* in SW480 increased the expression of both clock genes significantly ($p < 0.05$). Additionally, our analysis revealed a negative correlation between *MACC1* expression and two regulators of cancer and circadian rhythms, specifically *HRAS* and *SIRT1*. More interestingly, we reported for the first time that *MACC1* perturbations led to significant changes in the period, phase, and amplitude of circadian clock oscillations measured via *ARNTL*-promoter activity in living cells (**Figure 10C-D**). We found that *MACC1* overexpression increased the period of *ARNTL*-promoter activity, while *MACC1* KO decreased it. These changes were accompanied by alterations in cell proliferation, migration, and apoptosis upon *MACC1* manipulation (**Figure 10E-F**).

Altogether, our data propose a plausible reciprocal interplay between *MACC1* and the circadian clock machinery, contributing to observed perturbations in circadian rhythms and oncogenic progression, potentially mediated through core-clock elements.

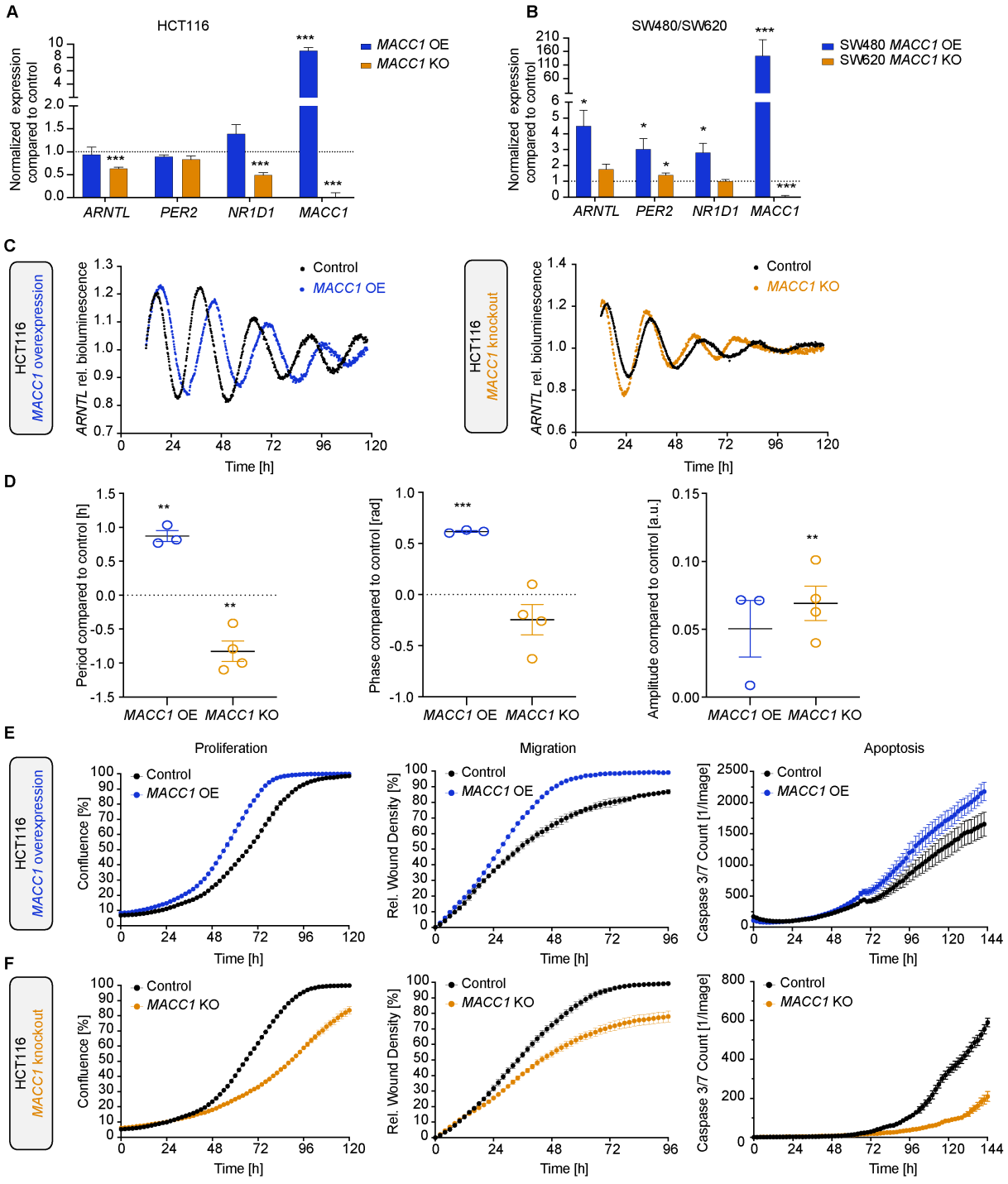

Figure 10: *MACC1* manipulation affects the circadian clock in CRC.

Gene expression analysis of core-clock genes *ARNTL*, *PER2* and *NR1D1* in *MACC1* manipulated (overexpression OE or knockout KO) (A) HCT116 or (B) SW480/SW620 cells. Samples were collected at 24h after synchronization (n = 3, mean ± SEM). (C-D) *ARNTL* promoter activity in *MACC1* manipulated HCT116 cell lines over five days using live-cell bioluminescence recordings. Period, phase and amplitude were measured using Chronostar and compared to control (n ≥ 3, mean ± SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed unpaired t-test. (E-F) Proliferation, migration and apoptosis analysis of HCT116 *MACC1* OE (E) and HCT116 *MACC1* KO (F) cell lines using live-cell imaging (n > 8, mean ± SEM, significance tested by comparing AUC with the respective control cell line, two-tailed unpaired t-test). Figure adapted from [88].

Rhythmic MACC1 protein expression is affected by clock alterations: a possible clock-MACC1 connection in CRC

To examine whether MACC1 is under circadian control, we measured MACC1 protein expression in a time-course manner in synchronized HCT116 cells. Remarkably, we found that MACC1 protein has a circadian rhythm that closely follows ARNTL rhythms and oscillates antiphase to NR1D1 rhythms in WT cells (**Figure 11A**). Interestingly, MACC1 circadian rhythms were disrupted in all core-clock knockout cell lines, suggesting the existence of a clock-MACC1 connection (**Figure 11B**). To test this, we investigated possible protein-protein interactions between the core-clock and MACC1 via co-immunoprecipitation assay. Indeed, we identified a MACC1-NR1D1 protein-protein interaction in HCT116 WT cells, underscoring a mechanistic link between the circadian clock and MACC1 at the protein level (**Figure 11C**). Notably, this interaction was lost after *NR1D1* depletion in HCT116 cells (**Figure 11D**).

Finally, we sought to investigate whether core-clock or *MACC1* alterations affect cell invasiveness in CRC (as an indirect indication of metastatic potential), by measuring chemotaxis 3D cell invasion over time. Our data showed that HCT116 cells with depleted core-clock elements (*ARNTL*, *PER2* or *NR1D1*) had increased invasive

capability compared to WT cells (**Figure 11E**). This effect was more prominent in *ARNTL* KO or *PER2* KO cells and similar to the *MACC1* overexpressing cell line. On the contrary, depletion of *MACC1* reduced invasiveness in HCT116 cells. Thus, our data revealed that core-clock depleted cells with disrupted MACC1 rhythms displayed increased invasiveness, suggesting that the interplay between *MACC1* and the circadian clock might regulate the progression of CRC cells, providing important insights into the molecular mechanisms underlying the circadian regulation of cancer metastasis in CRC.

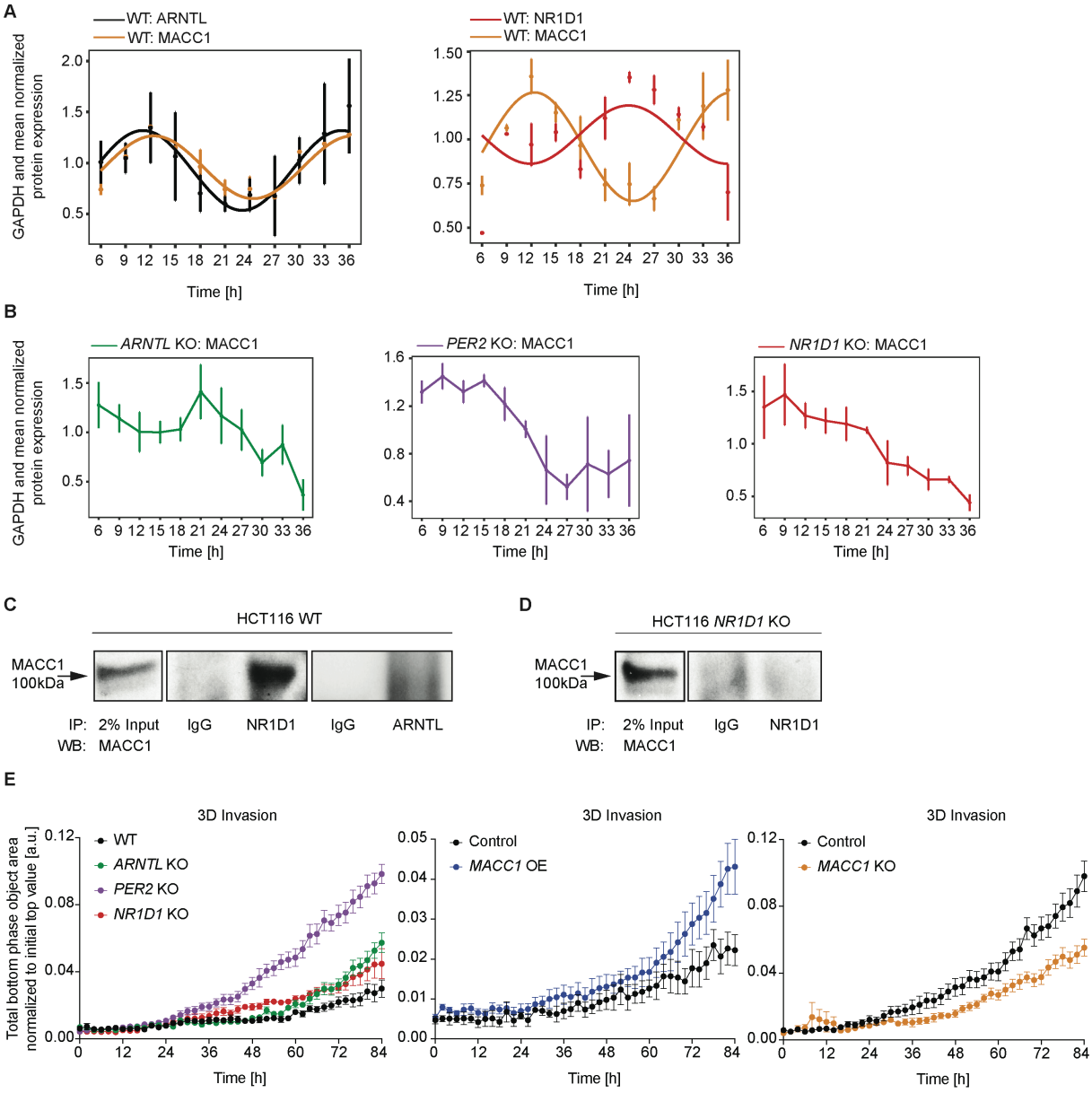

Figure 11: Circadian MACC1 oscillation is affected by core-clock manipulation in HCT116 cells.

30h time-course protein expression (ARNTL, NR1D1 or MACC1, western blotting) in synchronized (A) HCT116 WT cells. (B) MACC1 expression in synchronized *ARNTL* KO, *PER2* KO or *NR1D1* KO cells. GAPDH and mean normalized data are presented (n = 3, mean ± SEM). Rhythmicity analysis was performed using Cosinor in Discorhythm R package [92] for a period of 24h. A cosine curve was fitted for rhythmic data sets ($p < 0.05$), and data points were connected with closed lines for arrhythmic data points ($p > 0.05$). Co-IP analysis of MACC1 in (C) HCT116 WT and (D) *NR1D1* KO lysates. IP was performed for NR1D1 or ARNTL. Western blot was performed using MACC1 antibody. IgG: Isotype control. (E) 3D chemotaxis cell invasion analysis of HCT116 cells using IncuCyte S3 live-cell analysis within 84h (n = 8, mean ± SEM). Figure adapted from [88].

3.2.3 Discussion

In our second published work, we built upon and further investigated key findings from our previous work regarding the connection between the circadian clock and cancer invasion/metastasis potential in CRC. As previously reported, the circadian clock has been shown to play a role in cancer progression, including metastasis and metastatic potential in CRC. Low expression of *PER2* or *NR1D1* and upregulation of *CLOCK* have been found to be correlated with metastasis in CRC in patient samples and *in vivo* experiments [60,93,94]. Another study reported that the circadian core-clock element CRY1 was upregulated in CRC cell lines as well as in clinical samples, which correlated with increased cell migration *in vitro* and lymph node metastasis in patient samples [72]. Also, a recent study showed that *ARNTL* affects CRC progression and metastasis by stimulating exosome secretion *in vitro* [95]. Despite these findings, the mechanistic link between the circadian clock and CRC progression is still poorly understood and requires further investigation.

Here we showed that the circadian clock regulates elements related to cancer metastasis in CRC, particularly via *MACC1*, a gene associated with metastasis formation. MACC1 serves as a key mediator in metastatic pathways and plays an instrumental role in modulating therapeutic outcomes in CRC interventions [90,96]. We reported that the expression of *MACC1* is strongly affected by the disruption of the core-clock genes and showed for the first time that MACC1 is under circadian control and oscillates in phase with ARNTL rhythms in WT cells, which is lost after circadian clock disruption via core-clock KO. We further suggested that the clock-MACC1 connection may be mediated through *NR1D1*, for which we identified protein-protein interactions with MACC1, as seen by our immunoprecipitation assays. Interestingly,

we observed that the disruption of the circadian clock in CRC cell lines followed by disrupted MACC1 rhythms led to an increase in invasiveness potential, as measured by chemotaxis cell invasion through a 3D biomatrix. Specifically, cells with *PER2* KO exhibited a pronounced increase in cellular invasion, potentially attributed to a notable upregulation of *MACC1* and a decrease in *ECAD* expression. These alterations likely promote a more invasive cancer phenotype through the activation of EMT markers.

Strikingly, we also observed that *MACC1* manipulation (via overexpression or KO) affected core-clock gene expression as well as the circadian phenotype in CRC cells. We observed this effect by changes in the oscillation period of *ARNTL*-promoter activity upon *MACC1* manipulation, highlighting the existence of a potential bidirectional interplay between components of the circadian clock and *MACC1*. These data elucidate the integral function of core-clock genes in modulating CRC metastasis and invasion, potentially via an intricate molecular crosstalk with *MACC1* at both transcriptional and rhythmic oscillation levels. Given that MACC1 serves as a therapeutic target for CRC progression and metastasis [91], its circadian-mediated modulation is likely to influence treatment efficacy in a time-dependent manner. Therefore, our findings highlight a strong association between oncogenic properties like proliferation and invasion, and the circadian clock, mediated by *MACC1* in CRC.

However, further research is needed using primary cells from patient tumour samples and/or animal models to verify these interactions *in vivo* and determine their functional relevance in CRC progression as well as treatment.

4 General Discussion and Outlook

4.1 Circadian Clock in Cancer: Intertwined and interconnected

The circadian clock is an endogenous timekeeping system that orchestrates various physiological processes, including the sleep-wake cycle, metabolism, and immune function, to maintain proper homeostasis. It is regulated by a complex network of genes, proteins, and signalling pathways that function in feedback loops to generate and maintain robust rhythms in activity and behaviour. Disruption of the circadian clock has been linked to several health issues, including an increased risk of cancer formation and progression [97,98]. Circadian rhythm disruptions, such as shift work, jet lag, or exposure to light at night, have been associated with a higher incidence of several types of cancer, including breast, prostate, lung and colorectal cancer [99-102]. This is due to the fact that the circadian clock regulates multiple aspects of cell biology including the regulation of cell cycle and DNA repair mechanisms, control of metabolism as well as the modulation of immune function (reviewed in [61]), to name a few.

Interestingly, accumulating evidence points to the fact that cancer, in turn, can also impact the circadian clock through various mechanisms and thus supports the idea of a bidirectional interplay between the two elements. Cancer can affect the circadian clock on both systemic and molecular/cellular levels. These influences can disrupt the normal functioning of the circadian system and contribute to the progression of the disease. At the systemic level, cancer can disrupt circadian rhythms through the release of inflammatory cytokines (e.g., interleukin-6 and tumour necrosis factor-alpha) [103] and alterations in hormone levels (e.g., in breast cancer) or other signalling molecules [104,105]. These disruptions can affect various physiological processes,

such as sleep-wake cycles (e.g., leading to insomnia or fragmented sleep) [106] as well as changes in metabolism, all affecting the circadian clock function.

At the molecular and cellular level, cancer can affect the circadian clock by altering the expression or function of core-clock genes and proteins such as PERs, CRYs, CLOCK, and BMAL1 (reviewed in [52]). Animal studies have shown that tumours can impact circadian rhythms in tumour-free distal organs in breast, melanoma and lung cancer studies [107-109]. Other examples include the role of oncogenes and tumour suppressors, as seen for *MYC* and *TP53*, respectively, in affecting circadian clock function. It was shown that MYC increases the transcriptional activity of E-box sites and enhances *NR1D1/2* expression, which in turn inhibits BMAL1 function [110]. Another study showed that p53 plays a role in regulating the expression of *Per2* and maintaining the circadian rhythm in mice [67]. The authors demonstrated that p53 directly binds to the *Per2* promoter region, affecting its transcription as well as the mouse circadian behaviour. These alterations can further worsen the disruption of cellular processes, lead to disruptions in circadian-controlled pathways [111] and finally, contribute to cancer progression.

In the context of colorectal cancer (CRC), several studies have reported associations between clock gene expression and CRC development, progression, and prognosis (reviewed in [112]). For example, altered expression of clock genes, such as *ARNTL* and members of *PER* and *CRY* family have been observed in CRC tissues and colorectal liver metastases, and these alterations have been linked to clinicopathological features, including tumour stage, lymph node metastasis, and overall survival [69,113,114]. While some core-clock genes are reported to have tumour suppressor properties, others may promote CRC progression, though these findings are inconsistent and require further research. In addition, clinical studies in

humans have suggested that disruption of circadian rhythms may accelerate CRC progression and showed that circadian rhythms can be used as predictors of CRC patient survival [76,115]. Therefore, the circadian clock is thought to play a role in regulating CRC progression and metastasis, although the exact mechanisms are still unclear.

In our work, we showed evidence of molecular interplay between elements of the CCN and EMT related components in CRC with a profound impact in regulating cell proliferation, invasion and metastasis potential. Recent findings have highlighted the significance of core circadian clock genes (e.g., *BMAL1*) in EMT-induced CRC metastasis and drug resistance to chemotherapeutic agents [116]. Therefore, gaining a deeper understanding of core-clock genes in the epithelial-mesenchymal equilibrium of CRC cells could help develop strategies to reverse drug resistance in existing treatment methods. We further characterized the role of the circadian core-clock genes *BMAL1*, *PER2* and *NR1D1* in CRC progression and introduced a new EMT-related component, *MACC1,* as a mediator and effector of clock-cancer interplay. While *MACC1* manipulation affects core-clock rhythmicity and expression in CRC, disruptions of core-clock genes abolished MACC1 circadian rhythms and protein-protein interactions, and ultimately affected CRC invasiveness and metastasis ability, pointing towards a reciprocal complexity and interconnectivity between the two systems.

Further studies are needed to elucidate the precise molecular mechanisms linking the circadian clock, EMT and cancer metastasis, and to explore the potential benefits of chronotherapy and clock gene-targeted interventions in cancer management. Investigating this interaction could lead to the identification of novel therapeutic targets and improved prognosis, in addition to expanding our knowledge of cancer biology.

These findings may also help improve prognostic tools and patient stratification, allowing for more personalized approaches to cancer treatment. As a result, clinicians could tailor treatment strategies to individual patients, optimizing outcomes and potentially improving survival rates [117]. However, challenges remain in understanding the complex molecular mechanisms and translating findings from animal models to humans. This is mainly due to a) the complexity of the circadian system involving multiple genes, proteins, and signalling pathways, b) the inter-individual variability in circadian rhythms and c) difficulty in translating findings from animal models to humans due to species-specific differences in circadian regulation.

4.2 Towards Personalized Circadian Medicine

The implications of finding connections between the circadian clock and elements regulating cancer formation and metastasis are significant. Understanding the molecular mechanisms underlying these connections can provide valuable insights into cancer biology and potentially inform the development of novel therapeutic strategies. These include identifying novel drug targets, improving cancer prognosis, and ultimately utilizing personalized medicine. Indeed, these findings have led to the development of circadian medicine (i.e., chronotherapy) and chrono-oncology [41]. Chrono-oncology is a subfield of oncology that focuses on the study of the relationships between the circadian clock, cancer development, and cancer treatment. It investigates how disruptions in circadian rhythms can contribute to cancer development and progression, and how the circadian clock can be harnessed to improve cancer treatment efficacy and reduce side effects. By understanding the role of the circadian clock in cancer biology and applying this knowledge to the timing and administration of cancer therapies, chrono-oncology aims to improve patient outcomes

and quality of life. In general, circadian medicine and chrono-oncology (including in CRC) rely on three main strategies [41,56,118]:

a) Targeting the clock: Targeting circadian clock components and/or strengthening circadian rhythms using zeitgebers (e.g., light, food and exercise) could affect disease progression or even prevent their onset [119,120]. For example, pharmacological activation of REV-ERBs (via SR9009 and SR9011) and RORs (via nobiletin), two components of the core-clock network, has been reported to induce lethality in several cancers including CRC [82,121].

b) Timing of the drug: Since there is evidence of a clear time-of-day dependency in drug efficacy or toxicity shown in many pharmacological studies [41,122,123] this approach involves the timing of treatments according to daily rhythms in pharmacokinetics and pharmacodynamics, to reduce side effects and improve patient survival. For example, administering immunotherapy at specific times for melanoma patients has demonstrated better results compared to conventional treatment, both in reducing side effects and enhancing survival rates [124]. In CRC, chrono-modulated FOLFOX treatment in metastatic CRC patients resulted in a survival advantage compared to conventional treatment [75]. Indeed, a recent systematic review of 18 clinical studies involving 2547 cancer patients with various cancer types (e.g., colorectal, nasopharyngeal, endometrial and ovarian cancer) found that chrono-modulated chemotherapy significantly reduced treatment toxicity while maintaining efficacy in most cases [125].

c) Tracing the clock: As the effectiveness of therapeutic interventions can be dependent on an individual's internal circadian phase, treatments should be personalized according to the individual's chronotype (i.e., internal body clock). For successful circadian precision medicine, accurate, sensitive, reliable, cost-effective,

and practically applicable methods for assessing individual circadian phases and monitoring of biological rhythms are needed. While traditionally chronotype was determined through questionnaires, actigraphy, or examining the timing of evening melatonin rise in blood or saliva [126], newer, more practical biomarkers that use single blood or tissue samples are being considered (reviewed in [53]).

In a novel approach, we developed a non-invasive and user-friendly method to profile an individual's circadian rhythm using saliva samples collected over a 48-hour period during the daytime [127,128]. Our approach harnesses the molecular characterization of core-clock gene expressions, including *BMAL1* and *PER2*, combined with computational techniques like harmonic regression and mathematical modelling. This analysis produces an individual's circadian profile, characterized by amplitude, period, phase, and the mesor (mean level). This detailed individual circadian rhythm profile has several potential applications in both clinical and non-clinical settings. These include monitoring disease progression, optimizing the timing of medical treatments based on drug targets, and improving physical and cognitive performance, such as sports performance and sleep quality. In a pilot study using healthy subjects (15 participants, 7 women) we demonstrated that our method can be used to display inter-individual differences in circadian rhythms among participants, and can be utilized to predict individual times for best exercise performance using computational analysis [127]. Thus, our tool has the potential to optimize treatment timing in patients and enhance performance in healthy individuals.

Despite these and other advancements in translating circadian clock knowledge to health improvement and cancer treatment in patients, several challenges remain in finding routine applications based on a patient's circadian clock information. Future studies on the interplay between the circadian clock and cancer, particularly in colorectal cancer, should aim to deepen our understanding of the underlying molecular mechanisms, identify novel therapeutic targets, explore the role of the tumour microenvironment [129], and develop circadian-based biomarkers. By designing studies that address these areas, we can work towards improving patient outcomes and developing more effective and personalized treatments for colorectal cancer patients.

5 References

1. Edgar, R.S.; Green, E.W.; Zhao, Y.; van Ooijen, G.; Olmedo, M.; Qin, X.; Xu, Y.; Pan, M.; Valekunja, U.K.; Feeney, K.A., et al. Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **2012**, *485*, 459-464, doi:10.1038/nature11088.

2. Astaburuaga, R.; Basti, A.; Li, Y.; Herms, D.; Relógio, A. Circadian regulation of physiology: Relevance for space medicine. *Reach* **2019**, *14-15*, doi:10.1016/j.reach.2019.100029.

3. Zhang, R.; Lahens, N.F.; Ballance, H.I.; Hughes, M.E.; Hogenesch, J.B. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc Natl Acad Sci U S A* **2014**, *111*, 16219-16224, doi:10.1073/pnas.1408886111.

4. Mure, L.S.; Le, H.D.; Benegiamo, G.; Chang, M.W.; Rios, L.; Jillani, N.; Ngotho, M.; Kariuki, T.; Dkhissi-Benyahya, O.; Cooper, H.M., et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. *Science* **2018**, *359*, eaao0318, doi:10.1126/science.aao0318.

5. Lehmann, R.; Childs, L.; Thomas, P.; Abreu, M.; Fuhr, L.; Herzel, H.; Leser, U.; Relogio, A. Assembly of a comprehensive regulatory network for the mammalian circadian clock: a bioinformatics approach. *PLoS One* **2015**, *10*, e0126283, doi:10.1371/journal.pone.0126283.

6. Relogio, A.; Thomas, P.; Medina-Perez, P.; Reischl, S.; Bervoets, S.; Gloc, E.; Riemer, P.; Mang-Fatehi, S.; Maier, B.; Schafer, R., et al. Ras-mediated deregulation of the circadian clock in cancer. *PLoS Genet* **2014**, *10*, e1004338, doi:10.1371/journal.pgen.1004338.

7. Padmanabhan, K.; Robles, M.S.; Westerling, T.; Weitz, C.J. Feedback regulation of transcriptional termination by the mammalian circadian clock PERIOD complex. *Science* **2012**, *337*, 599-602, doi:10.1126/science.1221592.

8. Kang, T.H.; Reardon, J.T.; Sancar, A. Regulation of nucleotide excision repair activity by transcriptional and post-transcriptional control of the XPA protein. *Nucleic Acids Res* **2011**, *39*, 3176-3187, doi:10.1093/nar/gkq1318.

9. Aschoff, J. Circadian Control of Body-Temperature. *Journal of Thermal Biology* **1983**, *8*, 143-147, doi:Doi 10.1016/0306-4565(83)90094-3.

10. Green, C.B.; Takahashi, J.S.; Bass, J. The meter of metabolism. *Cell* **2008**, *134*, 728-742, doi:10.1016/j.cell.2008.08.022.

11. Levi, F.; Filipski, E.; Iurisci, I.; Li, X.M.; Innominato, P. Cross-talks between circadian timing system and cell division cycle determine cancer biology and therapeutics. *Cold Spring Harb Symp Quant Biol* **2007**, *72*, 465-475, doi:10.1101/sqb.2007.72.030.

12. Sahar, S.; Sassone-Corsi, P. Metabolism and cancer: the circadian clock connection. *Nat Rev Cancer* **2009**, *9*, 886-896, doi:10.1038/nrc2747.

13. Fuhr, L.; El-Athman, R.; Scrima, R.; Cela, O.; Carbone, A.; Knoop, H.; Li, Y.; Hoffmann, K.; Laukkanen, M.O.; Corcione, F., et al. The Circadian Clock Regulates Metabolic Phenotype Rewiring Via HKDC1 and Modulates Tumor Progression and Drug Response in Colorectal Cancer. *EBioMedicine* **2018**, *33*, 105-121, doi:10.1016/j.ebiom.2018.07.002.

14. El-Athman, R.; Genov, N.N.; Mazuch, J.; Zhang, K.; Yu, Y.; Fuhr, L.; Abreu, M.; Li, Y.; Wallach, T.; Kramer, A., et al. The Ink4a/Arf locus operates as a regulator of the circadian clock modulating RAS activity. *PLoS Biol* **2017**, *15*, e2002940, doi:10.1371/journal.pbio.2002940.

15. Abreu, M.; Basti, A.; Genov, N.; Mazzoccoli, G.; Relogio, A. The reciprocal interplay between TNFalpha and the circadian clock impacts on cell proliferation and migration in Hodgkin lymphoma cells. *Sci Rep* **2018**, *8*, 11474, doi:10.1038/s41598-018-29847-z.

16. Lamia, K.A. Ticking time bombs: connections between circadian clocks and cancer. *F1000Res* **2017**, *6*, 1910, doi:10.12688/f1000research.11770.1.

17. El-Athman, R.; Fuhr, L.; Relogio, A. A Systems-Level Analysis Reveals Circadian Regulation of Splicing in Colorectal Cancer. *EBioMedicine* **2018**, *33*, 68-81, doi:10.1016/j.ebiom.2018.06.012.

18. Genov, N.; Basti, A.; Abreu, M.; Relogio, A. Temporal Splicing Switches in Elements of the TNF-Pathway Identified by Computational Analysis of Transcriptome Data for Human Cell Lines. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20051182.

19. Genov, N.; Basti, A.; Abreu, M.; Astaburuaga, R.; Relogio, A. A bioinformatic analysis identifies circadian expression of splicing factors and time-dependent alternative splicing events in the HD-MY-Z cell line. *Sci Rep* **2019**, *9*, 11062, doi:10.1038/s41598-019-47343-w.

20. Malhan, D.; Basti, A.; Relogio, A. Transcriptome analysis of clock disrupted cancer cells reveals differential alternative splicing of cancer hallmarks genes. *NPJ Syst Biol Appl* **2022**, *8*, 17, doi:10.1038/s41540-022-00225-w.

21. Straif, K.; Baan, R.; Grosse, Y.; Secretan, B.; El Ghissassi, F.; Bouvard, V.; Altieri, A.; Benbrahim-Tallaa, L.; Cogliano, V.; Group, W.H.O.I.A.F.R.o.C.M.W. Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncol* **2007**, *8*, 1065-1066, doi:10.1016/S1470-2045(07)70373-X.

22. Scheiermann, C.; Kunisaki, Y.; Frenette, P.S. Circadian control of the immune system. *Nat Rev Immunol* **2013**, *13*, 190-198, doi:10.1038/nri3386.

23. Tosini, G.; Menaker, M. Circadian rhythms in cultured mammalian retina. *Science* **1996**, *272*, 419-421.

24. Yamazaki, S.; Numano, R.; Abe, M.; Hida, A.; Takahashi, R.; Ueda, M.; Block, G.D.; Sakaki, Y.; Menaker, M.; Tei, H. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **2000**, *288*, 682-685.

25. Abe, M.; Herzog, E.D.; Yamazaki, S.; Straume, M.; Tei, H.; Sakaki, Y.; Menaker, M.; Block, G.D. Circadian rhythms in isolated brain regions. *J Neurosci* **2002**, *22*, 350-356.

26. Gekakis, N.; Staknis, D.; Nguyen, H.B.; Davis, F.C.; Wilsbacher, L.D.; King, D.P.; Takahashi, J.S.; Weitz, C.J. Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **1998**, *280*, 1564-1569.

27. Yoo, S.H.; Ko, C.H.; Lowrey, P.L.; Buhr, E.D.; Song, E.J.; Chang, S.; Yoo, O.J.; Yamazaki, S.; Lee, C.; Takahashi, J.S. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. *Proc Natl Acad Sci U S A* **2005**, *102*, 2608-2613, doi:10.1073/pnas.0409763102.

28. Cho, H.; Zhao, X.; Hatori, M.; Yu, R.T.; Barish, G.D.; Lam, M.T.; Chong, L.W.; DiTacchio, L.; Atkins, A.R.; Glass, C.K., et al. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. *Nature* **2012**, *485*, 123-127, doi:10.1038/nature11048.

29. Stratmann, M.; Schibler, U. Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J Biol Rhythms* **2006**, *21*, 494-506, doi:10.1177/0748730406293889.

30. Dibner, C.; Schibler, U.; Albrecht, U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* **2010**, *72*, 517-549, doi:10.1146/annurev-physiol-021909-135821.

31. Dickmeis, T.; Weger, B.D.; Weger, M. The circadian clock and glucocorticoids--interactions across many time scales. *Mol Cell Endocrinol* **2013**, *380*, 2-15, doi:10.1016/j.mce.2013.05.012.

32. Kornmann, B.; Schaad, O.; Bujard, H.; Takahashi, J.S.; Schibler, U. System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol* **2007**, *5*, e34, doi:10.1371/journal.pbio.0050034.

33. Gachon, F.; Nagoshi, E.; Brown, S.A.; Ripperger, J.; Schibler, U. The mammalian circadian timing system: from gene expression to physiology. *Chromosoma* **2004**, *113*, 103-112, doi:10.1007/s00412-004-0296-2.

34. Sinturel, F.; Gos, P.; Petrenko, V.; Hagedorn, C.; Kreppel, F.; Storch, K.F.; Knutti, D.; Liani, A.; Weitz, C.; Emmenegger, Y., et al. Circadian hepatocyte clocks keep synchrony in the absence of a master pacemaker in the suprachiasmatic nucleus or other extrahepatic clocks. *Genes Dev* **2021**, *35*, 329-334, doi:10.1101/gad.346460.120.

35. Damiola, F.; Le Minh, N.; Preitner, N.; Kornmann, B.; Fleury-Olela, F.; Schibler, U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **2000**, *14*, 2950-2961, doi:10.1101/gad.183500.

36. Stokkan, K.A.; Yamazaki, S.; Tei, H.; Sakaki, Y.; Menaker, M. Entrainment of the circadian clock in the liver by feeding. *Science* **2001**, *291*, 490-493, doi:10.1126/science.291.5503.490.

37. Wolff, G.; Esser, K.A. Scheduled exercise phase shifts the circadian clock in skeletal muscle. *Med Sci Sports Exerc* **2012**, *44*, 1663-1670, doi:10.1249/MSS.0b013e318255cf4c.

38. Peek, C.B.; Levine, D.C.; Cedernaes, J.; Taguchi, A.; Kobayashi, Y.; Tsai, S.J.; Bonar, N.A.; McNulty, M.R.; Ramsey, K.M.; Bass, J. Circadian Clock Interaction with HIF1alpha Mediates Oxygenic Metabolism and Anaerobic Glycolysis in Skeletal Muscle. *Cell Metab* **2017**, *25*, 86-92, doi:10.1016/j.cmet.2016.09.010.

39. Patke, A.; Young, M.W.; Axelrod, S. Molecular mechanisms and physiological importance of circadian rhythms. *Nat Rev Mol Cell Biol* **2020**, *21*, 67-84, doi:10.1038/s41580-019-0179-2.

40. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144*, 646-674, doi:10.1016/j.cell.2011.02.013.

41. Cederroth, C.R.; Albrecht, U.; Bass, J.; Brown, S.A.; Dyhrfjeld-Johnsen, J.; Gachon, F.; Green, C.B.; Hastings, M.H.; Helfrich-Forster, C.; Hogenesch, J.B., et al. Medicine in the Fourth Dimension. *Cell Metab* **2019**, *30*, 238-250, doi:10.1016/j.cmet.2019.06.019.

42. Gaucher, J.; Montellier, E.; Sassone-Corsi, P. Molecular Cogs: Interplay between Circadian Clock and Cell Cycle. *Trends Cell Biol* **2018**, *28*, 368-379, doi:10.1016/j.tcb.2018.01.006.

43. Bernard, S.; Herzel, H. Why do cells cycle with a 24 hour period? *Genome Inform* **2006**, *17*, 72-79.

44. Masri, S.; Cervantes, M.; Sassone-Corsi, P. The circadian clock and cell cycle: interconnected biological circuits. *Curr Opin Cell Biol* **2013**, *25*, 730-734, doi:10.1016/j.ceb.2013.07.013.

45. Bieler, J.; Cannavo, R.; Gustafson, K.; Gobet, C.; Gatfield, D.; Naef, F. Robust synchronization of coupled circadian and cell cycle oscillators in single mammalian cells. *Mol Syst Biol* **2014**, *10*, 739, doi:10.15252/msb.20145218.

46. Miyazaki, K.; Wakabayashi, M.; Hara, Y.; Ishida, N. Tumor growth suppression in vivo by overexpression of the circadian component, PER2. *Genes Cells* **2010**, *15*, 351-358, doi:10.1111/j.1365-2443.2010.01384.x.

47. Zeng, Z.L.; Wu, M.W.; Sun, J.; Sun, Y.L.; Cai, Y.C.; Huang, Y.J.; Xian, L.J. Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity. *J Biochem* **2010**, *148*, 319-326, doi:10.1093/jb/mvq069.

48. Feillet, C.; van der Horst, G.T.; Levi, F.; Rand, D.A.; Delaunay, F. Coupling between the Circadian Clock and Cell Cycle Oscillators: Implication for Healthy Cells and Malignant Growth. *Front Neurol* **2015**, *6*, 96, doi:10.3389/fneur.2015.00096.

49. Matsu-Ura, T.; Dovzhenok, A.; Aihara, E.; Rood, J.; Le, H.; Ren, Y.; Rosselot, A.E.; Zhang, T.; Lee, C.; Obrietan, K., et al. Intercellular Coupling of the Cell Cycle and Circadian Clock in Adult Stem Cell Culture. *Mol Cell* **2016**, *64*, 900-912, doi:10.1016/j.molcel.2016.10.015.

50. Gery, S.; Komatsu, N.; Baldjyan, L.; Yu, A.; Koo, D.; Koeffler, H.P. The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. *Mol Cell* **2006**, *22*, 375-382, doi:10.1016/j.molcel.2006.03.038.

51. Lin, Z.; Fang, D. The Roles of SIRT1 in Cancer. *Genes Cancer* **2013**, *4*, 97-104, doi:10.1177/1947601912475079.

52. Kinouchi, K.; Sassone-Corsi, P. Metabolic rivalry: circadian homeostasis and tumorigenesis. *Nat Rev Cancer* **2020**, *20*, 645-661, doi:10.1038/s41568-020-0291-9.

53. Hesse, J.; Malhan, D.; Yalin, M.; Aboumanify, O.; Basti, A.; Relogio, A. An Optimal Time for Treatment-Predicting Circadian Time by Machine Learning and Mathematical Modelling. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12113103.

54. Levi, F. From circadian rhythms to cancer chronotherapeutics. *Chronobiol Int* **2002**, *19*, 1-19.

55. Sancar, A.; Van Gelder, R.N. Clocks, cancer, and chronochemotherapy. *Science* **2021**, *371*, 42-49, doi:10.1126/science.abb0738.

56. Sulli, G.; Manoogian, E.N.C.; Taub, P.R.; Panda, S. Training the Circadian Clock, Clocking the Drugs, and Drugging the Clock to Prevent, Manage, and Treat Chronic Diseases. *Trends Pharmacol Sci* **2018**, *39*, 812-827, doi:10.1016/j.tips.2018.07.003.

57. Shafi, A.A.; Knudsen, K.E. Cancer and the Circadian Clock. *Cancer Res* **2019**, *79*, 3806-3814, doi:10.1158/0008-5472.Can-19-0566.

58. Brown, S.A.; Fleury-Olela, F.; Nagoshi, E.; Hauser, C.; Juge, C.; Meier, C.A.; Chicheportiche, R.; Dayer, J.M.; Albrecht, U.; Schibler, U. The period length of fibroblast circadian gene expression varies widely among human individuals. *PLoS Biol* **2005**, *3*, e338, doi:10.1371/journal.pbio.0030338.

59. Maier, B.; Lorenzen, S.; Finger, A.M.; Herzel, H.; Kramer, A. Searching Novel Clock Genes Using RNAi-Based Screening. *Methods Mol Biol* **2021**, *2130*, 103-114, doi:10.1007/978-1-0716-0381-9_8.

60. Basti, A.; Fior, R.; Yalin, M.; Povoa, V.; Astaburuaga, R.; Li, Y.; Naderi, J.; Godinho Ferreira, M.; Relogio, A. The Core-Clock Gene NR1D1 Impacts Cell Motility In Vitro and Invasiveness in A Zebrafish Xenograft Colon Cancer Model. *Cancers (Basel)* **2020**, *12*, doi:10.3390/cancers12040853.

61. Sulli, G.; Lam, M.T.Y.; Panda, S. Interplay between Circadian Clock and Cancer: New Frontiers for Cancer Treatment. *Trends Cancer* **2019**, *5*, 475-494, doi:10.1016/j.trecan.2019.07.002.

62. Deb, S.P.; Singh, S.; Deb, S. MDM2 overexpression, activation of signaling networks, and cell proliferation. *Subcell Biochem* **2014**, *85*, 215-234, doi:10.1007/978-94-017-9211-0_12.

63. Ferrara, N.; Mass, R.D.; Campa, C.; Kim, R. Targeting VEGF-A to treat cancer and age-related macular degeneration. *Annu Rev Med* **2007**, *58*, 491-504, doi:10.1146/annurev.med.58.061705.145635.

64. Feuers, R.J.; Casciano, D.A.; Tsai, T.H.; Scheving, L.E. Regulation of the circadian rhythm of hepatic pyruvate kinase in mice. *Prog Clin Biol Res* **1987**, *227A*, 163-172.

65. Li, Y.; Basti, A.; Yalcin, M.; Relogio, A. Circadian Dysregulation of the TGFbeta/SMAD4 Pathway Modulates Metastatic Properties and Cell Fate Decisions in Pancreatic Cancer Cells. *iScience* **2020**, *23*, 101551, doi:10.1016/j.isci.2020.101551.

66. Fuhr, L.; Abreu, M.; Pett, P.; Relogio, A. Circadian systems biology: When time matters. *Comput Struct Biotechnol J* **2015**, *13*, 417-426, doi:10.1016/j.csbj.2015.07.001.

67. Miki, T.; Matsumoto, T.; Zhao, Z.; Lee, C.C. p53 regulates Period2 expression and the circadian clock. *Nat Commun* **2013**, *4*, 2444, doi:10.1038/ncomms3444.

68. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **2021**, *71*, 209-249, doi:10.3322/caac.21660.

69. Mazzoccoli, G.; Panza, A.; Valvano, M.R.; Palumbo, O.; Carella, M.; Pazienza, V.; Biscaglia, G.; Tavano, F.; Di Sebastiano, P.; Andriulli, A., et al. Clock gene expression levels and relationship with clinical and pathological features in colorectal cancer patients. *Chronobiol Int* **2011**, *28*, 841-851, doi:10.3109/07420528.2011.615182.

70. Zeng, Z.L.; Luo, H.Y.; Yang, J.; Wu, W.J.; Chen, D.L.; Huang, P.; Xu, R.H. Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer. *Clin Cancer Res* **2014**, *20*, 1042-1052, doi:10.1158/1078-0432.CCR-13-0171.

71. Huber, A.L.; Papp, S.J.; Chan, A.B.; Henriksson, E.; Jordan, S.D.; Kriebs, A.; Nguyen, M.; Wallace, M.; Li, Z.; Metallo, C.M., et al. CRY2 and FBXL3 Cooperatively Degrade c-MYC. *Mol Cell* **2016**, *64*, 774-789, doi:10.1016/j.molcel.2016.10.012.

72. Yu, H.; Meng, X.; Wu, J.; Pan, C.; Ying, X.; Zhou, Y.; Liu, R.; Huang, W. Cryptochrome 1 overexpression correlates with tumor progression and poor prognosis in patients with colorectal cancer. *PLoS One* **2013**, *8*, e61679, doi:10.1371/journal.pone.0061679.

73. Levi, F.; Zidani, R.; Misset, J.L. Randomised multicentre trial of chronotherapy with oxaliplatin, fluorouracil, and folinic acid in metastatic colorectal cancer. International Organization for Cancer Chronotherapy. *Lancet* **1997**, *350*, 681-686, doi:10.1016/s0140-6736(97)03358-8.

74. Innominato, P.F.; Karaboue, A.; Focan, C.; Chollet, P.; Giacchetti, S.; Bouchahda, M.; Ulusakarya, A.; Torsello, A.; Adam, R.; Levi, F.A., et al. Efficacy and safety of chronomodulated irinotecan, oxaliplatin, 5-fluorouracil and leucovorin combination as first- or second-line treatment against metastatic colorectal cancer: Results from the International EORTC 05011 Trial. *Int J Cancer* **2020**, *148*, 2512-2521, doi:10.1002/ijc.33422.

75. Giacchetti, S.; Bjarnason, G.; Garufi, C.; Genet, D.; Iacobelli, S.; Tampellini, M.; Smaaland, R.; Focan, C.; Coudert, B.; Humblet, Y., et al. Phase III trial comparing 4-day chronomodulated therapy versus 2-day conventional delivery of fluorouracil, leucovorin, and oxaliplatin as first-line chemotherapy of metastatic colorectal cancer: the European Organisation for Research and Treatment of Cancer Chronotherapy Group. *J Clin Oncol* **2006**, *24*, 3562-3569, doi:10.1200/JCO.2006.06.1440.

76. Levi, F.; Dugue, P.A.; Innominato, P.; Karaboue, A.; Dispersyn, G.; Parganiha, A.; Giacchetti, S.; Moreau, T.; Focan, C.; Waterhouse, J., et al. Wrist actimetry circadian rhythm as a robust predictor of colorectal cancer patients survival. *Chronobiol Int* **2014**, *31*, 891-900, doi:10.3109/07420528.2014.924523.

77. Innominato, P.F.; Komarzynski, S.; Palesh, O.G.; Dallmann, R.; Bjarnason, G.A.; Giacchetti, S.; Ulusakarya, A.; Bouchahda, M.; Haydar, M.; Ballesta, A., et al. Circadian rest-activity rhythm as an objective biomarker of patient-reported outcomes in patients with advanced cancer. *Cancer Med* **2018**, *7*, 4396-4405, doi:10.1002/cam4.1711.

78. Innominato, P.; Komarzynski, S.; Karaboue, A.; Ulusakarya, A.; Bouchahda, M.; Haydar, M.; Bossevot-Desmaris, R.; Mocquery, M.; Plessis, V.; Levi, F. Home-Based e-Health Platform for Multidimensional Telemonitoring of Symptoms, Body Weight, Sleep, and Circadian Activity: Relevance for Chronomodulated Administration of Irinotecan, Fluorouracil-Leucovorin, and Oxaliplatin at Home-Results From a Pilot Study. *JCO Clin Cancer Inform* **2018**, *2*, 1-15, doi:10.1200/CCI.17.00125.

79. Fior, R.; Povoa, V.; Mendes, R.V.; Carvalho, T.; Gomes, A.; Figueiredo, N.; Ferreira, M.G. Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts. *Proc Natl Acad Sci U S A* **2017**, *114*, E8234-E8243, doi:10.1073/pnas.1618389114.

80. Povoa, V.; Rebelo de Almeida, C.; Maia-Gil, M.; Sobral, D.; Domingues, M.; Martinez-Lopez, M.; de Almeida Fuzeta, M.; Silva, C.; Grosso, A.R.; Fior, R. Innate immune evasion revealed in a colorectal zebrafish xenograft model. *Nat Commun* **2021**, *12*, 1156, doi:10.1038/s41467-021-21421-y.

81. Wood, P.A.; Yang, X.; Taber, A.; Oh, E.Y.; Ansell, C.; Ayers, S.E.; Al-Assaad, Z.; Carnevale, K.; Berger, F.G.; Pena, M.M., et al. Period 2 mutation accelerates ApcMin/+ tumorigenesis. *Mol Cancer Res* **2008**, *6*, 1786-1793, doi:10.1158/1541-7786.MCR-08-0196.

82. Sulli, G.; Rommel, A.; Wang, X.; Kolar, M.J.; Puca, F.; Saghatelian, A.; Plikus, M.V.; Verma, I.M.; Panda, S. Pharmacological activation of REV-ERBs is lethal in cancer and oncogene-induced senescence. *Nature* **2018**, *553*, 351-355, doi:10.1038/nature25170.

83. Zhang, H.; Shu, R.; Liu, X.; Zhang, X.; Sun, D. Downregulation of REV-ERBalpha is associated with the progression of lung adenocarcinoma. *Ann Transl Med* **2022**, *10*, 56, doi:10.21037/atm-21-6405.

84. Wang, S.; Li, F.; Lin, Y.; Wu, B. Targeting REV-ERBalpha for therapeutic purposes: promises and challenges. *Theranostics* **2020**, *10*, 4168-4182, doi:10.7150/thno.43834.

85. Thiery, J.P.; Acloque, H.; Huang, R.Y.; Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* **2009**, *139*, 871-890, doi:10.1016/j.cell.2009.11.007.

86. Wang, Y.; Kojetin, D.; Burris, T.P. Anti-proliferative actions of a synthetic REV-ERBalpha/beta agonist in breast cancer cells. *Biochem Pharmacol* **2015**, *96*, 315-322, doi:10.1016/j.bcp.2015.06.010.

87. Tao, L.; Yu, H.; Liang, R.; Jia, R.; Wang, J.; Jiang, K.; Wang, Z. Rev-erbalpha inhibits proliferation by reducing glycolytic flux and pentose phosphate pathway in human gastric cancer cells. *Oncogenesis* **2019**, *8*, 57, doi:10.1038/s41389-019-0168-5.

88. Basti, A.; Malhan, D.; Dumbani, M.; Dahlmann, M.; Stein, U.; Relogio, A. Core-Clock Genes Regulate Proliferation and Invasion via a Reciprocal Interplay with MACC1 in Colorectal Cancer Cells. *Cancers (Basel)* **2022**, *14*, doi:10.3390/cancers14143458.

89. Innominato, P.; Komarzynski, S.; Karaboué, A.; Ulusakarya, A.; Bouchahda, M.; Haydar, M.; Bossevot-Desmaris, R.; Mocquery, M.; Plessis, V.; Lévi, F. Home-Based e-Health Platform for Multidimensional Telemonitoring of Symptoms, Body Weight, Sleep, and Circadian Activity: Relevance for Chronomodulated Administration of Irinotecan, Fluorouracil-Leucovorin, and Oxaliplatin at Home—Results From a Pilot Study. *JCO Clinical Cancer Informatics* **2018**, 10.1200/cci.17.00125, 1-15, doi:10.1200/cci.17.00125.

90. Stein, U.; Walther, W.; Arlt, F.; Schwabe, H.; Smith, J.; Fichtner, I.; Birchmeier, W.; Schlag, P.M. MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis. *Nat Med* **2009**, *15*, 59-67, doi:10.1038/nm.1889.

91. Radhakrishnan, H.; Walther, W.; Zincke, F.; Kobelt, D.; Imbastari, F.; Erdem, M.; Kortum, B.; Dahlmann, M.; Stein, U. MACC1-the first decade of a key metastasis molecule from gene discovery to clinical translation. *Cancer Metastasis Rev* **2018**, *37*, 805-820, doi:10.1007/s10555-018-9771-8.

92. Carlucci, M.; Krisciunas, A.; Li, H.; Gibas, P.; Koncevicius, K.; Petronis, A.; Oh, G. DiscoRhythm: an easy-to-use web application and R package for discovering rhythmicity. *Bioinformatics* **2021**, *36*, 1952–1954, doi:10.1093/bioinformatics/btab517.

93. Oshima, T.; Takenoshita, S.; Akaike, M.; Kunisaki, C.; Fujii, S.; Nozaki, A.; Numata, K.; Shiozawa, M.; Rino, Y.; Tanaka, K., et al. Expression of circadian genes correlates with liver metastasis and outcomes in colorectal cancer. *Oncol Rep* **2011**, *25*, 1439-1446, doi:10.3892/or.2011.1207.

94. Wang, Y.; Sun, N.; Lu, C.; Bei, Y.; Qian, R.; Hua, L. Upregulation of circadian gene 'hClock' contribution to metastasis of colorectal cancer. *Int J Oncol* **2017**, *50*, 2191-2199, doi:10.3892/ijo.2017.3987.

95. Dong, P.; Wang, Y.; Liu, Y.; Zhu, C.; Lin, J.; Qian, R.; Hua, L.; Lu, C. BMAL1 induces colorectal cancer metastasis by stimulating exosome secretion. *Mol Biol Rep* **2022**, *49*, 373-384, doi:10.1007/s11033-021-06883-z.

96. Juneja, M.; Kobelt, D.; Walther, W.; Voss, C.; Smith, J.; Specker, E.; Neuenschwander, M.; Gohlke, B.O.; Dahlmann, M.; Radetzki, S., et al. Statin and rottlerin small-molecule

inhibitors restrict colon cancer progression and metastasis via MACC1. *PLoS Biol* **2017**, *15*, e2000784, doi:10.1371/journal.pbio.2000784.

97. Lee, Y. Roles of circadian clocks in cancer pathogenesis and treatment. *Exp Mol Med* **2021**, *53*, 1529-1538, doi:10.1038/s12276-021-00681-0.

98. Pariollaud, M.; Lamia, K.A. Cancer in the Fourth Dimension: What Is the Impact of Circadian Disruption? *Cancer Discov* **2020**, *10*, 1455-1464, doi:10.1158/2159-8290.CD-20-0413.

99. Wegrzyn, L.R.; Tamimi, R.M.; Rosner, B.A.; Brown, S.B.; Stevens, R.G.; Eliassen, A.H.; Laden, F.; Willett, W.C.; Hankinson, S.E.; Schernhammer, E.S. Rotating Night-Shift Work and the Risk of Breast Cancer in the Nurses' Health Studies. *Am J Epidemiol* **2017**, *186*, 532-540, doi:10.1093/aje/kwx140.

100. Flynn-Evans, E.E.; Mucci, L.; Stevens, R.G.; Lockley, S.W. Shiftwork and prostate-specific antigen in the National Health and Nutrition Examination Survey. *J Natl Cancer Inst* **2013**, *105*, 1292-1297, doi:10.1093/jnci/djt169.

101. Papagiannakopoulos, T.; Bauer, M.R.; Davidson, S.M.; Heimann, M.; Subbaraj, L.; Bhutkar, A.; Bartlebaugh, J.; Vander Heiden, M.G.; Jacks, T. Circadian Rhythm Disruption Promotes Lung Tumorigenesis. *Cell Metab* **2016**, *24*, 324-331, doi:10.1016/j.cmet.2016.07.001.

102. Wang, X.; Ji, A.; Zhu, Y.; Liang, Z.; Wu, J.; Li, S.; Meng, S.; Zheng, X.; Xie, L. A meta-analysis including dose-response relationship between night shift work and the risk of colorectal cancer. *Oncotarget* **2015**, *6*, 25046-25060, doi:10.18632/oncotarget.4502.

103. Arjona, A.; Sarkar, D.K. Evidence supporting a circadian control of natural killer cell function. *Brain Behav Immun* **2006**, *20*, 469-476, doi:10.1016/j.bbi.2005.10.002.

104. Allende, S.; Medina, J.L.; Spiegel, D.; Zeitzer, J.M. Evening salivary cortisol as a single stress marker in women with metastatic breast cancer. *Psychoneuroendocrinology* **2020**, *115*, 104648, doi:10.1016/j.psyneuen.2020.104648.

105. Sephton, S.E.; Sapolsky, R.M.; Kraemer, H.C.; Spiegel, D. Diurnal cortisol rhythm as a predictor of breast cancer survival. *J Natl Cancer Inst* **2000**, *92*, 994-1000, doi:10.1093/jnci/92.12.994.

106. Innominato, P.F.; Spiegel, D.; Ulusakarya, A.; Giacchetti, S.; Bjarnason, G.A.; Levi, F.; Palesh, O. Subjective sleep and overall survival in chemotherapy-naive patients with metastatic colorectal cancer. *Sleep Med* **2015**, *16*, 391-398, doi:10.1016/j.sleep.2014.10.022.

107. Hojo, H.; Enya, S.; Arai, M.; Suzuki, Y.; Nojiri, T.; Kangawa, K.; Koyama, S.; Kawaoka, S. Remote reprogramming of hepatic circadian transcriptome by breast cancer. *Oncotarget* **2017**, *8*, 34128-34140, doi:10.18632/oncotarget.16699.

108. de Assis, L.V.M.; Moraes, M.N.; Magalhaes-Marques, K.K.; Kinker, G.S.; da Silveira Cruz-Machado, S.; Castrucci, A.M.L. Non-Metastatic Cutaneous Melanoma Induces

Chronodisruption in Central and Peripheral Circadian Clocks. *Int J Mol Sci* **2018**, *19*, doi:10.3390/ijms19041065.

109. Masri, S.; Papagiannakopoulos, T.; Kinouchi, K.; Liu, Y.; Cervantes, M.; Baldi, P.; Jacks, T.; Sassone-Corsi, P. Lung Adenocarcinoma Distally Rewires Hepatic Circadian Homeostasis. *Cell* **2016**, *165*, 896-909, doi:10.1016/j.cell.2016.04.039.

110. Altman, B.J.; Hsieh, A.L.; Sengupta, A.; Krishnanaiah, S.Y.; Stine, Z.E.; Walton, Z.E.; Gouw, A.M.; Venkataraman, A.; Li, B.; Goraksha-Hicks, P., et al. MYC Disrupts the Circadian Clock and Metabolism in Cancer Cells. *Cell Metab* **2015**, *22*, 1009-1019, doi:10.1016/j.cmet.2015.09.003.

111. Fu, L.; Kettner, N.M. The circadian clock in cancer development and therapy. *Prog Mol Biol Transl Sci* **2013**, *119*, 221-282, doi:10.1016/B978-0-12-396971-2.00009-9.

112. Rao, X.; Lin, L. Circadian clock as a possible control point in colorectal cancer progression (Review). *Int J Oncol* **2022**, *61*, doi:10.3892/ijo.2022.5439.

113. Mazzoccoli, G.; Colangelo, T.; Panza, A.; Rubino, R.; De Cata, A.; Tiberio, C.; Valvano, M.R.; Pazienza, V.; Merla, G.; Augello, B., et al. Deregulated expression of cryptochrome genes in human colorectal cancer. *Mol Cancer* **2016**, *15*, 6, doi:10.1186/s12943-016-0492-8.

114. Huisman, S.A.; Ahmadi, A.R.; JN, I.J.; Verhoef, C.; van der Horst, G.T.; de Bruin, R.W. Disruption of clock gene expression in human colorectal liver metastases. *Tumour Biol* **2016**, *37*, 13973-13981, doi:10.1007/s13277-016-5231-7.

115. Innominato, P.F.; Focan, C.; Gorlia, T.; Moreau, T.; Garufi, C.; Waterhouse, J.; Giacchetti, S.; Coudert, B.; Iacobelli, S.; Genet, D., et al. Circadian rhythm in rest and activity: a biological correlate of quality of life and a predictor of survival in patients with metastatic colorectal cancer. *Cancer Res* **2009**, *69*, 4700-4707, doi:10.1158/0008-5472.CAN-08-4747.

116. Zhang, Y.; Devocelle, A.; Desterke, C.; de Souza, L.E.B.; Hadadi, E.; Acloque, H.; Foudi, A.; Xiang, Y.; Ballesta, A.; Chang, Y., et al. BMAL1 Knockdown Leans Epithelial-Mesenchymal Balance toward Epithelial Properties and Decreases the Chemoresistance of Colon Carcinoma Cells. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22105247.

117. Kramer, A.; Lange, T.; Spies, C.; Finger, A.M.; Berg, D.; Oster, H. Foundations of circadian medicine. *PLoS Biol* **2022**, *20*, e3001567, doi:10.1371/journal.pbio.3001567.

118. Battaglin, F.; Chan, P.; Pan, Y.; Soni, S.; Qu, M.; Spiller, E.R.; Castanon, S.; Roussos Torres, E.T.; Mumenthaler, S.M.; Kay, S.A., et al. Clocking cancer: the circadian clock as a target in cancer therapy. *Oncogene* **2021**, *40*, 3187-3200, doi:10.1038/s41388-021-01778-6.

119. Panda, S. The arrival of circadian medicine. *Nat Rev Endocrinol* **2019**, *15*, 67-69, doi:10.1038/s41574-018-0142-x.

120. Ruan, W.; Yuan, X.; Eltzschig, H.K. Circadian rhythm as a therapeutic target. *Nat Rev Drug Discov* **2021**, *20*, 287-307, doi:10.1038/s41573-020-00109-w.

121. Ashrafizadeh, M.; Zarrabi, A.; Saberifar, S.; Hashemi, F.; Hushmandi, K.; Hashemi, F.; Moghadam, E.R.; Mohammadinejad, R.; Najafi, M.; Garg, M. Nobiletin in Cancer Therapy: How This Plant Derived-Natural Compound Targets Various Oncogene and Onco-Suppressor Pathways. *Biomedicines* **2020**, *8*, 31, doi:10.3390/biomedicines8050110.

122. Ruben, M.D.; Smith, D.F.; FitzGerald, G.A.; Hogenesch, J.B. Dosing time matters. *Science* **2019**, *365*, 547-549, doi:10.1126/science.aax7621.

123. Gorbacheva, V.Y.; Kondratov, R.V.; Zhang, R.; Cherukuri, S.; Gudkov, A.V.; Takahashi, J.S.; Antoch, M.P. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc Natl Acad Sci U S A* **2005**, *102*, 3407-3412, doi:10.1073/pnas.0409897102.

124. Qian, D.C.; Kleber, T.; Brammer, B.; Xu, K.M.; Switchenko, J.M.; Janopaul-Naylor, J.R.; Zhong, J.; Yushak, M.L.; Harvey, R.D.; Paulos, C.M., et al. Effect of immunotherapy time-of-day infusion on overall survival among patients with advanced melanoma in the USA (MEMOIR): a propensity score-matched analysis of a single-centre, longitudinal study. *Lancet Oncol* **2021**, *22*, 1777-1786, doi:10.1016/S1470-2045(21)00546-5.

125. Printezi, M.I.; Kilgallen, A.B.; Bond, M.J.G.; Stibler, U.; Putker, M.; Teske, A.J.; Cramer, M.J.; Punt, C.J.A.; Sluijter, J.P.G.; Huitema, A.D.R., et al. Toxicity and efficacy of chronomodulated chemotherapy: a systematic review. *Lancet Oncol* **2022**, *23*, e129-e143, doi:10.1016/S1470-2045(21)00639-2.

126. Reiter, A.M.; Sargent, C.; Roach, G.D. Finding DLMO: estimating dim light melatonin onset from sleep markers derived from questionnaires, diaries and actigraphy. *Chronobiol Int* **2020**, *37*, 1412-1424, doi:10.1080/07420528.2020.1809443.

127. Basti, A.; Yalçin, M.; Herms, D.; Hesse, J.; Aboumanify, O.; Li, Y.; Aretz, Z.; Garmshausen, J.; El-Athman, R.; Hastermann, M., et al. Diurnal variations in the expression of core-clock genes correlate with resting muscle properties and predict fluctuations in exercise performance across the day. *BMJ Open Sport & Exercise Medicine* **2021**, *7*, doi:10.1136/bmjsem-2020-000876.

128. Dose, B.; Yalcin, M.; Dries, S.P.M.; Relogio, A. TimeTeller for timing health: The potential of circadian medicine to improve performance, prevent disease and optimize treatment. *Front Digit Health* **2023**, *5*, 1157654, doi:10.3389/fdgth.2023.1157654.

129. Xuan, W.; Khan, F.; James, C.D.; Heimberger, A.B.; Lesniak, M.S.; Chen, P. Circadian regulation of cancer cell and tumor microenvironment crosstalk. *Trends Cell Biol* **2021**, *31*, 940-950, doi:10.1016/j.tcb.2021.06.008.

130. Murgo, E.; De Santis, E.; Sansico, F.; Melocchi, V.; Colangelo, T.; Padovano, C.; Colucci, M.; Carbone, A.; Totti, B.; Basti, A., et al. The circadian clock circuitry

modulates leukemia initiating cell activity in T-cell acute lymphoblastic leukemia. *J Exp Clin Cancer Res* **2023**, *42*, 218, doi:10.1186/s13046-023-02799-7.

131. Ludwig, M.; Basti, A.; Yalcin, M.; Schulte, J.H.; Relogio, A. Molecular characterization of the circadian clock in paediatric leukaemia patients: a prospective study protocol. *BMC Pediatr* **2023**, *23*, 105, doi:10.1186/s12887-023-03921-6.

132. Fuhr, L.; Basti, A.; Bras, T.S.; Duarte, M.F.; Relogio, A. Antiproliferative Effects of Cynara Cardunculus in Colorectal Cancer Cells Are Modulated by the Circadian Clock. *Int J Mol Sci* **2022**, *23*, doi:10.3390/ijms23169130.

133. Yalcin, M.; Malhan, D.; Basti, A.; Peralta, A.R.; Ferreira, J.J.; Relogio, A. A Computational Analysis in a Cohort of Parkinson's Disease Patients and Clock-Modified Colorectal Cancer Cells Reveals Common Expression Alterations in Clock-Regulated Genes. *Cancers (Basel)* **2021**, *13*, doi:10.3390/cancers13235978.

134. Abreu, M.; Basti, A.; Genov, N.; Mazzoccoli, G.; Relógio, A. The reciprocal interplay between TNFα and the circadian clock impacts on cell proliferation and migration in Hodgkin lymphoma cells. *Scientific Reports* **2018**, *8*, 11474, doi:10.1038/s41598-018-29847-z.

6 List of Publications

The work presented in this thesis was developed between December 2017 and October 2022 in the group of Prof. Dr. Angela Relógio (Systems Biology of Cancer) at the Institute for Theoretical Biology (ITB) and the Molecular Cancer Research Centre (MKFZ) Charité - Universitätsmedizin Berlin, Humboldt-Universität zu Berlin. The work was funded by the German Federal Ministry of Education and Research (BMBF, eBio-CIRSPLICE, FKZ031A316), the Rolf M. Schwiete Stiftung and by the Berlin School of Integrative Oncology (BSIO) of the Charité - Universitätsmedizin Berlin.

Parts of this dissertation have been published [60,88].

6.1 First Authorships

6.1.1 Core-clock genes regulate proliferation and invasion via a reciprocal interplay with MACC1 in colorectal cancer cells [88]

6.1.2 Diurnal variations in the expression of core-clock genes correlate with resting muscle properties and predict fluctuations in exercise performance across the day [127]

6.1.3 The Core-Clock Gene NR1D1 Impacts Cell Motility In Vitro and Invasiveness in a Zebrafish Xenograft Colon Cancer Model [60]

6.2 Co-Authorships

6.2.1 The circadian clock circuitry modulates leukemia initiating cell activity in T-cell acute lymphoblastic leukemia [130]

6.2.2 Molecular characterization of the circadian clock in paediatric leukaemia patients: a prospective study protocol [131]

6.2.3 Antiproliferative Effects of Cynara Cardunculus in Colorectal Cancer Cells Are Modulated by the Circadian Clock [132]

6.2.4 Transcriptome analysis of clock disrupted cancer cells reveals differential alternative splicing of cancer hallmarks genes [20]

6.2.5 A Computational Analysis in a Cohort of Parkinson's Disease Patients and Clock-Modified Colorectal Cancer Cells Reveals Common Expression Alterations in Clock-Regulated Genes [133]

6.2.6 An optimal time for treatment—predicting circadian time by machine learning and mathematical modelling (review article) [53]

6.2.7 Circadian dysregulation of the TGFβ/SMAD4 pathway modulates metastatic properties and cell fate decisions in pancreatic cancer cells [65]

6.2.8 A bioinformatic analysis identifies circadian expression of splicing factors and time-dependent alternative splicing events in the HD-MY-Z cell line [19]

6.2.9 Circadian regulation of physiology: Relevance for space medicine (review article) [2]

6.2.10 Temporal splicing switches in elements of the TNF-pathway identified by computational analysis of transcriptome data for human cell lines [18]

6.2.11 The reciprocal interplay between TNFα and the circadian clock impacts on cell proliferation and migration in Hodgkin lymphoma cells [134]

Selbstständigkeitserklärung

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I hereby declare that I completed the doctoral thesis independently based on the stated resources and aids. I have not applied for a doctoral degree elsewhere and do not have a corresponding doctoral degree. I have not submitted the doctoral thesis, or parts of it, to another academic institution and the thesis has not been accepted or rejected. I declare that I have acknowledged the Doctoral Degree Regulations which underlie the procedure of the Faculty of Life Sciences of Humboldt-Universität zu Berlin, as amended on 05.03.2015. Furthermore, I declare that no collaboration with commercial doctoral degree supervisors took place, and that the principles of Humboldt-Universität zu Berlin for ensuring good academic practice were abided by.