DISSERTATION

A comprehensive bioinformatic analysis of circadian regulation of cellular pathways in an *in-vitro* model of colorectal cancer (CRC) and neurodegeneration

Eine umfassende bioinformatische Analyse der zirkadianen Regulierung zellulärer Signalwege in einem in-vitro-Modell für Kolorektalkarzinom (CRC) und Neurodegeneration

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von

Müge Yalçin

aus Izmir, Turkei

Erstbetreuerin: Prof. Dr. Angela Relógio

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1. Abstract

At the heart of the universe lies the synchrony of nature at every scale, including in our bodies. Building upon this concept Nobel Prize in Physiology or Medicine in 2017 was awarded to the discovery of the circadian clock which is the fascinating molecular genetic mechanism that allows organisms to anticipate, respond, adjust, and synchronize with recurring periodic events. The human circadian system, which is essential for fine-tuning the timing of biological events around the 24-hour solar day, comprises a network of clocks distributed throughout the body, with the suprachiasmatic nucleus in the brain acting as a central regulator. Any disruption to cellular clocks or disruption of their synchrony can lead to alterations in cellular pathways such as cell cycle or DNA repair.

This doctoral dissertation aimed to characterize expression changes at the transcriptomic level upon disruption of core-clock genes on common cellular pathways associated with cancer and neurodegeneration (ND). High-throughput data sets retrieved from idiopathic Parkinson's disease (IPD) patients (438 samples) and an *in vitro* colorectal carcinoma (CRC), including Wild Type (WT) and clock Knock Out (KO) cells (64 samples) were analyzed. The gene expression changes observed in individuals with IPD were similar to the patterns seen in core-clock KO cells. Curated literature and KEGG pathways searches were used to determine a set of relevant pathways and their corresponding involved genes in ND and cancer. Among those genes we found genes associated with genetic forms of PD such as *SNCA, GBA*, and core-clock regulator *DBP*. Notably, co-expression of core-clock network was altered in IPD patients compared to healthy controls, pointing to variations in the expression profile of PD-associated genes that may result from the disruption of the core-clock (as observed *in vitro* in the CRC model). Additionally, our subsequent analysis using the cancer genome atlas (TCGA) database revealed that genes, identified in our analysis, belong to cancer hallmark pathways and had a significant impact on clinical traits such as overall survival in colon cancer patients. Researchers have just started to shed light on the consequences of the disruptions in circadian rhythms, which could contribute to a deeper understanding of the molecular mechanisms underlying pathological development. Our results further point to a common regulation of specific pathways in cancer and ND and highlight the importance of the circadian system in this regulation. By characterizing these oscillations, an overlooked feature in the clinical routines, may actually reveal molecular signatures that can improve the diagnosis and monitoring of these pathologies.

1.1. Zusammenfassung

Dem Universums liegt eine Synchronität der Natur in jedem Bereich zugrunde, auch dem menschlichen Körper. Basierend auf diesem Konzept wurde die Entdeckung des faszinierenden molekulargenetischen Mechanismus der zirkadianen Uhr, mit dem Nobelpreis im Jahr 2017 gewürdigt. Diese ermöglicht Organismen periodisch wiederkehrende Prozesse zu antizipieren, darauf zu reagieren, sich anzupassen und zu synchronisieren. Das zirkadiane System des Menschen, das für die zeitliche Anpassung biologischer Ereignisse an den 24-Stunden-Tag notwendig ist, besteht aus einem Netzwerk molekularer Uhren, welches im ganzen Körper verteilt ist und in dem der suprachiasmatische Nukleus im Gehirn als Hauptregulator funktioniert. Jede Störung der zellulären Uhren oder ihrer Synchronität kann zu Veränderungen zellulärer Signalwege wie z.B. dem Zellzyklus oder der DNA-Reparatur führen.

Ziel dieser Dissertation war es, Veränderungen herkömmlicher zellulärer Prozesse auf Transkriptom-Ebene zu charakterisieren, welche durch Störung des zirkadianen Systems auftreten und mit Krebs und Neurodegeneration (ND) in Verbindung stehen. Zu diesem Zweck wurden Hochdurchsatzdaten von Patienten mit idiopathischer Parkinson-Krankheit (IPD) (438 Proben), sowie von einem in-vitro-Modell des kolorektalen Karzinoms (CRC), einschließlich Wildtyp (WT)- und zirkadiane Knock-Out (KO)-Zellen, (64 Proben) analysiert. Mehrere Genexpressionsveränderungen der IPD-Patienten ähnelten Expressionsprofilen der CRC zirkadianen KO-Zellen. Mithilfe einer kuratierten Literatur- und KEGG-Suche wurden eine Reihe relevanter Signalwege und die daran beteiligten Gene bei ND und Krebs ermittelt. Einige dieser Gene sind dafür bekannt mit einer genetischen Formen der Parkinson-Krankheit in Verbindung zu stehen, z.B. *SNCA*, *GBA* und das Core-Clock-Gen *DBP*. Im Vergleich zu gesunden Kontrollpersonen, wurden bei Parkinson-Patienten insbesondere Veränderungen des Core-Clock-Netzwerks festgestellt. Dies weist darauf hin, dass Variationen der Expressionsprofile Parkinson-assoziierter Gene möglicherweise aus der Störung von Core-Clock-Genen resultieren (wie in-vitro im CRC-Modell beobachtet). Darüber hinaus ergab unsere anschließende Analyse mit Hilfe der The Cancer-Genome Atlas (TCGA) Datenbank, dass die identifizierten Gene zu Krebs-Hallmark-Signalwegen gehören und einen signifikanten Einfluss auf klinische Eigenschaften wie die Überlebensdauer bei Darmkrebspatienten haben. Als Wissenschaftler stehen wir noch am Anfang klinische Auswirkungen von Störungen des zirkadianen Systems zu erforschen - in Zukunft wird dies jedoch zu einem

besseren Verständnis molekularer Mechanismen von Krankheitsentwicklung beitragen. Unsere Ergebnisse stützen die Hypothese einer gemeinsamen Regulierung bestimmter Signalwege bei Krebs und ND und unterstreichen die dabei wichtige Rolle des zirkadianen Systems. Da die Rolle zirkadianer Rhythmik in der klinischen Routine noch oft übersehen wird, könnte deren Charakterisierung durch Identifikation entsprechender molekular Signaturen tatsächlich die Diagnose und Überwachung dieser Pathologien verbessern.

2. Introduction

2.1. The clocks that underlie biological timing - an introduction to the mammalian circadian system

The necessity for an organism to accurately anticipate; respond and adjust to recurring periodic events favoured the emergence of “biological clocks” ticking to rule the daily lives. The human circadian system has evolved to fine-tune the appropriate timing of biological events around 24-hour (h) solar day and thereby termed circadian (*circa* - about, *diem* – a day) clock (1). This temporal regulation ensures the internal synchrony in behavior and physiology. Prominent examples are the control of sleep-wake cycle, with the release of the hormone melatonin promoting sleep at night and its suppression during the day promoting wakefulness; body temperature, which is lowest during the night, metabolism, and immune function, all of which exhibit daily rhythmic patterns (2). The circadian system is comprised of network of clocks distributed throughout the body (virtually in every cell) and the suprachiasmatic nucleus (SCN) positioned at the base of hypothalamus acting as a central pacemaker. The neuro-hormonal network conveys signals detected from the environment to all peripheral clocks through the SCN **(Figure 1A)**. The circadian rhythms (CRs) can be reset via external (e.g., light) timing cues called “Zeitgebers” (1, 3). The daily light exposure act as a reliable and dominant periodic signal to reset the human CR (4), but other Zeitgebers may interfere with circadian oscillations such as nutrient availability (5) or physical exercise (6) **(Figure 1A)**.

CRs are output of Transcriptional Translational Feedback Loops (TTFLs) involving a network of genes and proteins (7). In humans, at the beginning of each circadian cycle, positive limb of the core TTFL, namely Brain and Muscle ARNT-Like 1 (ARNTL; also known as BMAL1) and Circadian Locomotor Output Cycles Protein Kaput (CLOCK) heterodimerize and induce the expression of a multitude of genes, including their own repressors Period (PER) and Cryptochrome (CRY). At dawn, accumulated PER and CRY transit to the nucleus, where they suppress the activity of ARNTL and CLOCK **(Figure 1B).** Following degradation of PER–CRY heterodimer complexes, a new circadian cycle begins. To maintain the robust activity, a secondary loop is composed of Retinoic Acid Receptor-Related Orphan Receptor (ROR) activators and REV-ERB repressors from the nuclear receptor subfamily 1 group D member 1 and 2 (NR1D1 and NR1D2 also known as REV-ERB-α and REV-ERB-β) acts on *ARNTL* transcription. These interconnected components work together to produce ~ 24 h rhythmic oscillations and regulate temporal expression of clock-controlled genes (CCGs) **(Figure 1B)**. Rhythmic activity of the core-clock and CCGs regulate important cellular processes including DNA repair (8), metabolism (9, 10) , cellular growth (11) and death (12).

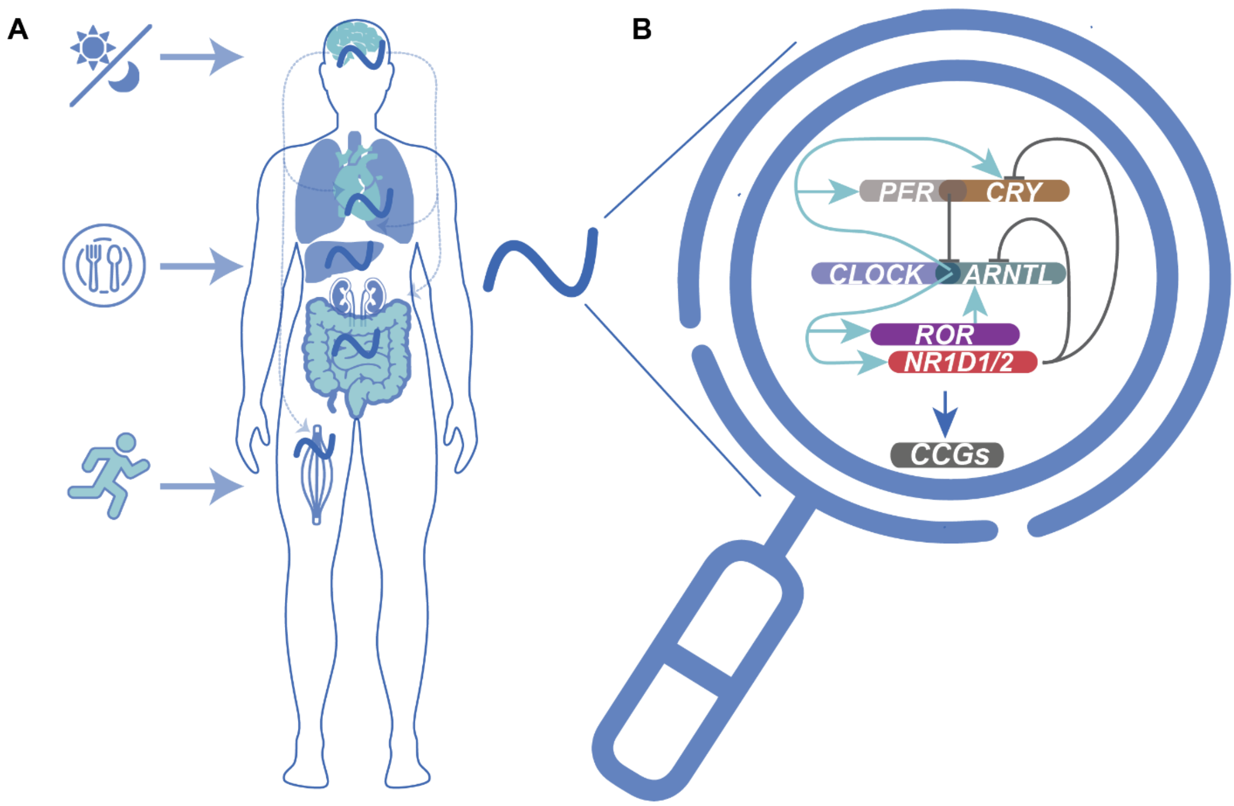

Figure 1. The components of human circadian system. (A) Upon exposure to cues such as daily light-dark cycles, meals, or physical exercise, also known as Zeitgebers, the circadian clock is reset. Light triggers a specialized set of neurons called "photosensitive ganglion cells" to transmit information to the central pacemaker of the clock machinery – the SCN which then synchronizes peripheral clocks throughout the body using neuronal, molecular, endocrine, or behavioral outputs. **(B)** Circadian Rhythms are generated via self-sustained feedback loops of positive and negative core-clock genes and proteins, including ARNTL, CLOCK, PERs, CRYs, RORs, and REV-ERBs (NR1D1, and NR1D2). The core-clock genes also regulate the circadian expression of Clock Controlled Genes (CCGs) (**Figure modified from Yalçin, M**., Mundorf, A., Thiel, F., Amatriain-Fernández, S., Kalthoff, I. S., Beucke, J. C., Budde, H., Garthus-Niegel, S., Peterburs, J., & Relógio, A. (2022). It's About Time: The Circadian Network as Time-Keeper for Cognitive Functioning, Locomotor Activity and Mental Health. Frontiers in Physiology (13), **refer to Figure 2 in the publication** (13)).

2.2. The hidden clock: uncovering the circadian system's impact on disease

Evidence from the recent years suggests that disruption of the circadian rhythms (CRs) can anticipate a role in the pathological development. Such disruptions can be caused by genetic factors altering the activity level of the clock or CCGs, or by environmental factors, such as long-term shift work (**Figure 2A**). Under normal physiological conditions, all peripheral tissue clocks are synchronized to the daily cycle according to the light signal received from the external environment. The SCN receives the photic information and transmits neuro-humoral signals to ensure the adjustment of phases between internal and external time a process known as 'entrainment' (14). Constant alteration of the timing of daily activities, for example, change in meal times, can result in the circadian misalignment. Inappropriate artificial light exposure and/or changed meal timings results in long-term circadian desynchrony between the SCN and peripheral clocks and may disrupt circadian outputs such as sleep and wake cycle (**Figure 2A**). The circadian disruption was linked to a range of diseases including sleep and mood disorders (15, 16), neurodegenerative diseases (17) and cancer (18) (**Figure 2A**). Although the evidence supports the involvement of circadian disruption in pathological development, the implementation of circadian medicine in clinical settings is still limited. However, the study of circadian rhythms has the potential to provide new avenues for treating and preventing diseases, which could ultimately lead to improved clinical applications.

Available standard therapies have been partially successful in order to eliminate cancer cells via chemotherapy, but the therapy success varies heavily as it might be terminated due to observed severe side effects, or fails to function due to acquired therapy resistance. The frequency of disease relapses underscores the variability in treatment outcomes resulting from both inter-patient and intra-patient differences (19). As a result, cancer research has shifted its focus towards developing personalized treatment plans that account for these individual variations. Recently, a promising association between the circadian clock and crucial molecular mechanisms that play a pivotal role in the tumorigenesis has been unveiled (20). This connection presents a potential breakthrough in personalized cancer therapy, as incorporating CRs into the design of treatment regimens could lead to more effective outcomes (21). New and exciting approaches are emerging, such as non-pharmacological interventions, to address circadian disruptions in clinical settings. These interventions include the use of physical exercise and light therapy, with circadian lighting solutions being implemented in hospital intensive care units (ICUs) (22). Reverting circadian disruptions through bright light therapy also showed a positive impact on motor and non-motor symptoms of several neurodegenerative disorders including Alzheimer's Disease (AD) and Parkinson's Disease (PD) and patients with dementia (23, 24) suggesting a role for circadian clock to improve disease management.

Yet, ranging from inadequacy of circadian rhythm assessment methods to difficulty of integrating circadian medicine into clinical schedule due to the complexity of introducing time as a component in the planning of studies reflected an extreme discrepancy between development of chronobiology at basic research and clinical level (**Figure 2B**). Assessment of CR at gene expression level requires collection of several samples over a time course consequently making it hard for patients to comply, and results in high experimental costs and causing another obstacle for implementation of circadian biology in clinical settings. Thus, monitoring and profiling the core-clock and CCGs expression is extremely relevant to identify novel drug targets and to optimize the timing of drug or therapeutic delivery. To demonstrate the disparities between fundamental chronobiology research and clinical studies in the field, we conducted a PubMed search using MeSH terms for circadian clock and chronotherapy over a 10-year period (2011-2021) (13). The low number of clinical studies in the field reflects the lack of consideration for circadian biology in clinical study protocols (**Figure 2B**). Despite the significant growth in chronobiology at basic research level, its translation into clinical practice for health maintenance and disease prevention remains scarce as evident in the limited number of clinical studies on topic (**Figure 2B**).

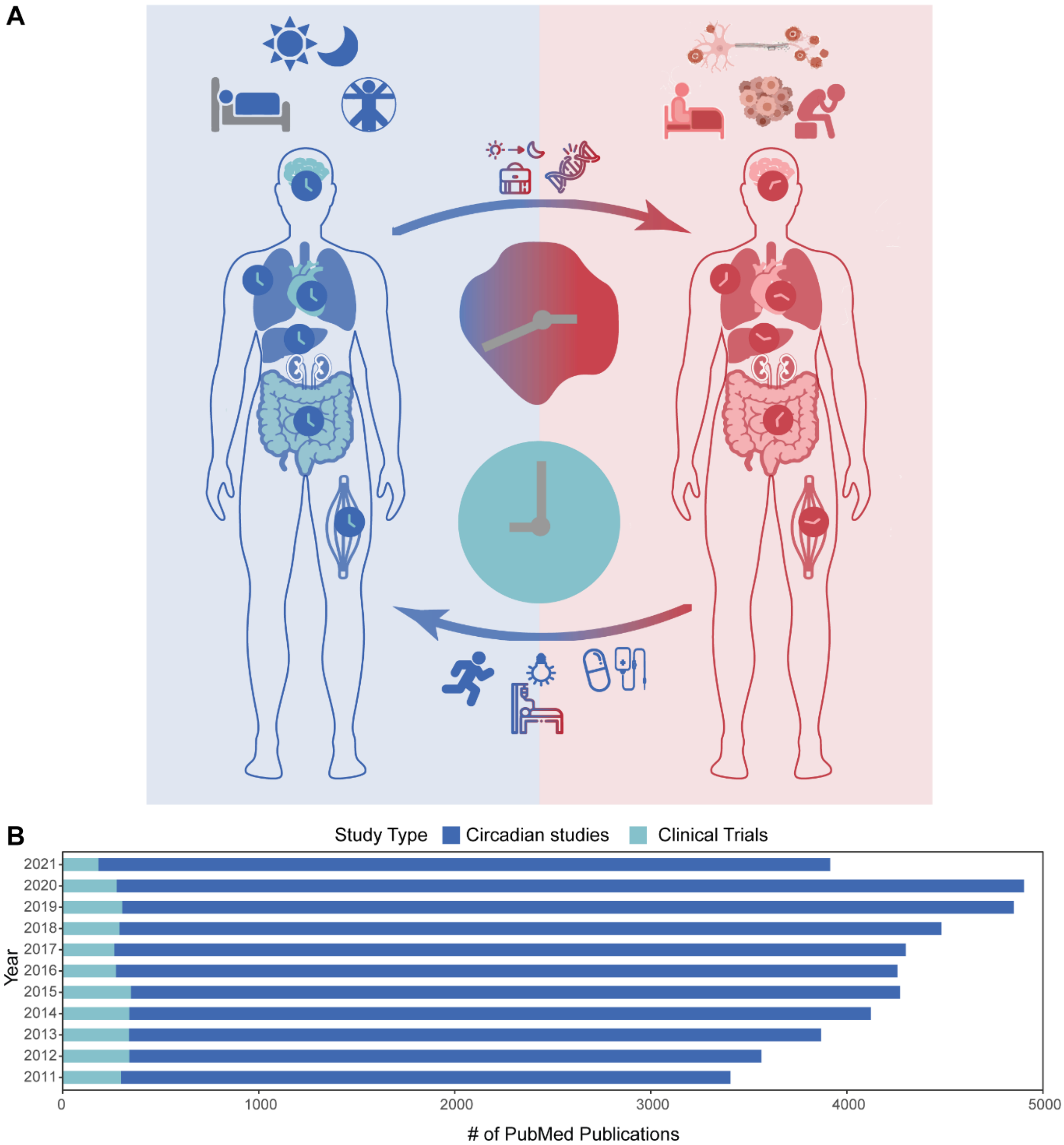

Figure 2. The circadian regulation impacts human physiology and disease. **(A)** The circadian clocks ensure physiological and behavioural events take place at the appropriate time interval such as sleep and wake cycles (left panel). Circadian rhythms can be disrupted by genetic factors or lifestyle e.g., long-term shift work. The clock disruption is linked to various disorders ranging from mood to behavior and sleep disorders, neurodegenerative diseases (ND) and cancer (right panel). **(B)** The last 10-year PubMed literature search shows an increasing trend in terms of number of publications focused on the circadian research (studies with basic research) denoted in light blue. The clinical studies denoted in dark blue (clinical trials) indicates studies in literature in which chronotherapy was included in the study protocol. (**Figure retrieved from Yalçin, M.,** Mundorf, A., Thiel, F., Amatriain-Fernández, S., Kalthoff, I. S., Beucke, J. C., Budde, H., Garthus-Niegel, S., Peterburs, J., & Relógio, A. (2022). It's About Time: The Circadian Network as Time-Keeper for Cognitive Functioning, Locomotor Activity and Mental Health. Frontiers in Physiology (13), **refer to Figure 1 and 3 in publication** (13)).

2.3. The time connection: unveiling the link between cancer and neurodegeneration

Cancer and neurodegenerative disorders are a major public health concern affecting around in total 40 million people in 2020 according to studies, which investigated the burden of these pathologies across most prevalent 36 cancer types and 13 neurological disorders (25, 26). Aging is linked to both pathologies (27) and known to impact the circadian system (28). With aging, the proper functioning of circadian rhythms is disrupted in part due to the diminished sensitivity of the SCN neurons to light stimulation. This consequently may lead to a misalignment of internal circadian rhythms with respect to the external light/dark cycles in the environment which can contribute to these pathologies (29). Cancer and neurodegeneration (ND) are traditionally put into opposite poles of disease spectrum: (i) due to the resistance of cancer cells to apoptosis whereas increased neuronal loss being a key cellular process in ND; (ii) discrepancy in terms of differential regulation of gene expression in critical disease associated markers (e.g., *P53*, a well-known tumor suppressor gene (30), inactivated in approximately 50% of human cancers and upregulated in ND in the brain samples from Alzheimer's Disease (AD) and Parkinson's Disease (PD) patients as well as in mouse models (31)) and (iii) epidemiological studies which pointed to an inverse correlation among cancer incidence and neurodegenerative disease patients (**Figure 3A**).

In recent years, published data has highlighted overlap between these two pathologies at the molecular level (32, 33). Several studies reported ND patients are at a higher risk to develop certain types of tumors: for example, PD patients tend to develop melanoma (34-36) and brain cancer (37, 38) more commonly compared to controls although an exact causal relationship is not elucidated. Both cancer and ND involve a multistep accumulation of erroneous cellular processes, including aberrant activity of key elements in cellular pathways such as DNA repair, cell cycle, redox homeostasis and immune system, which impact the onset and advancement of both pathologies (**Figure 3A**) and are regulated by the clock (8, 11, 39-43).

The circadian clock can reset upon adjustment to short-term misalignments, for example, when one travels across several time zones, an event known as jet lag. Yet, the evidence from population scale studies suggested constant disruption of circadian dynamics are associated with an increased risk of developing certain cancer types among shift workers such as breast cancer (44, 45) (**Figure 3B, left panel**). The disruption of circadian dynamics was also implicated in ND (46, 47) as evidenced by the worsening of patient's motor and non-motor symptoms at late hours of the day also called as 'Sundowning Effect', which has been linked to a change in rhythmic regulation of core-body temperature - a direct output of the circadian clock, in AD patients (48) (**Figure 3B, right panel**). Recently, several lines of work pointed to a role for the circadian regulation in ND-associated pathways in context of cancer *in-vitro* cellular models (49, 50). Our recent work using time course datasets (51) derived from colorectal cancer (CRC) SW480 (ATCC CCL-228) and SW620 (ATCC CCL-227) cells collected at distinct stages of tumor (primary tumor SW480 cells and SW620 cells, which is the metastatic counterpart and derived from the same patient) revealed changes in the rhythmicity patterns of neurodegeneration-associated pathways pointing to the involvement of circadian system in this link, which we further investigated in the scope of this thesis. In the following section, the role of circadian clock in regulation of cellular pathways, that are tightly linked to disrupted mechanisms in ND and cancer, which is the focus of this thesis will be introduced.

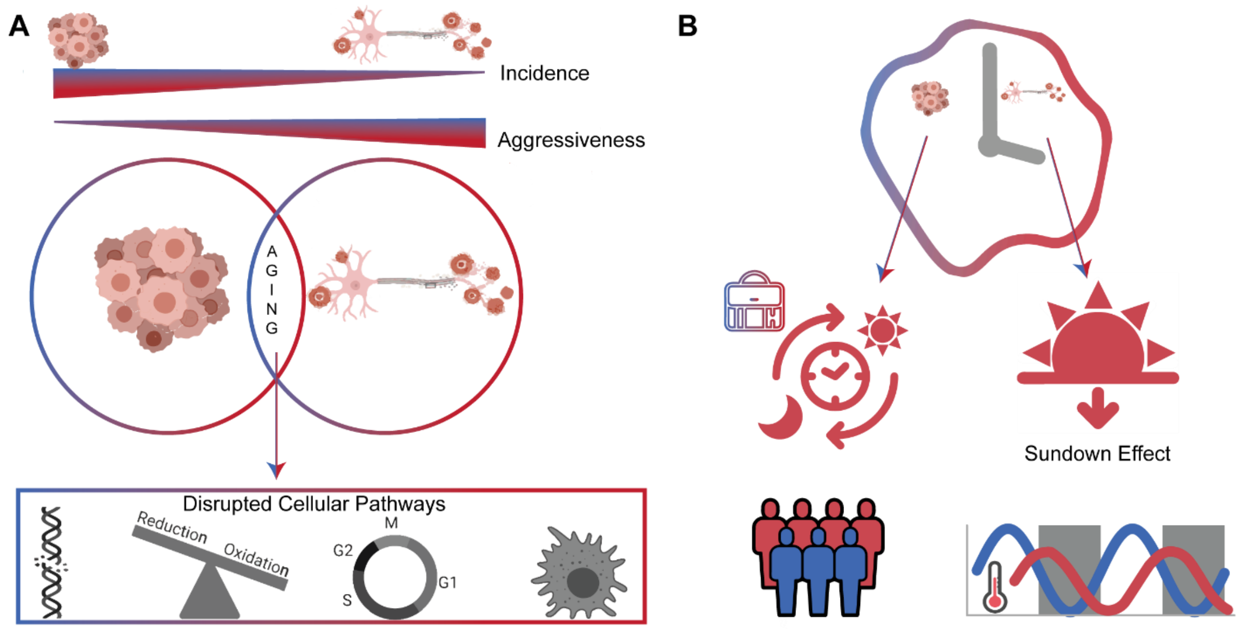

Figure 3. The circadian clock as an emerging link connecting neurodegeneration and cancer. (A) The inverse correlation between cancer and ND incidence places these pathologies to opposite poles of disease spectrum. The aging being a risk factor in both, commonly disrupted cellular pathways in cancer and ND are DNA repair, redox balance, cell cycle and immune system. **(B)** The disruption of circadian system linked to both cancer and neurodegenerative diseases. As a result of constant disruption of circadian rhythms, long term shift workers are suggested to be more prone to certain types of cancer shown in population scale studies. The disruption of circadian dynamics was also implicated in ND, for example,

by worsening of patient’s motor and non-motor symptoms at late hours of the day also called as ‘Sundowning Effect’.

2.3.1. Circadian regulation of cellular processes in tumorigenesis

The hallmarks of cancer refer to biological processes that have been reprogrammed by tumors to promote the survival and growth of cancer cells (52, 53). The circadian clock is known to regulate several of these hallmarks (54) including regulation of the DNA damage repair, apoptosis and cell cycle. The cell cycle, which is a commonly disrupted process both in ND and several cancer models, for example, is known to be tightly regulated by the circadian clock. It can interfere with cell cycle via several routes from modulation of cell cycle checkpoints (e.g., by interacting with *Wee1* kinase (55)), *Myc* (56, 57) or *p53* (58). Furthermore, it has been observed that when core-clock genes are disturbed, it can lead to a higher risk of tumor development such as the *Per2* gene, regarded as a tumor suppressor in several mouse models, including colorectal cancer (CRC) (59), lymphoma (60) and oral squamous cell carcinoma (61).

CRC is the third death-leading and the second most prevalent cancer globally (62). In 2020 alone, there has been more than 1.8 million new cases, which is expected to be increased in the upcoming decades (25). In the context of chrono-modulated therapeutic administration of chemotherapeutic drugs, CRC is one of the best studied examples although further studies are required to elucidate potential of optimization for an individual therapeutic scheme considering each patient’s personal circadian rhythm. Several clinical studies showed a benefit from chrono-modulated administration of conventional first-line chemotherapeutic drugs in CRC patients, including irinotecan (63, 64), 5-fluorouracil (65) and oxaliplatin (66). However, various results observed in clinical measures evaluating the effectiveness of chronotherapy in different subgroups of patients indicate the influence of other clinical factors such as sex, age and stage on treatment outcomes (67-69). These factors should be investigated further in the future to improve the characterization of clinical categories. For example, chrono-modulated administration showed a better outcome among men compared to women in CRC (70). One potential explanation for such a diverse outcome could be due to sex-dependent regulation of circadian genes that result in differential alteration of genes, such as *ATP-binding cassette (ABC) transporters Subfamily B Member 1* (*ABCB1)* as they are involved in absorption or other metabolic processes of drug and therapeutic compounds (71).

Transcriptomic approaches are crucial for investigating circadian related disturbances, as they allow for the identification and analysis of circadian gene expression alterations in response to disruptions in core-clock elements. In the context of this thesis, to study cellular alterations upon core-clock disruption in commonly altered cancer and ND pathways, a computational analysis of time series transcriptomics was performed on datasets derived from HCT116-wild type (WT) CRC cells and CRISPR-Cas9 generated-core-clock knockout (KO) cells (HCT116-*ARNTL*^{KO}; HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} cells) representing alteration of different clock components. By conducting the analysis with KO cells, we aimed to mimic the alteration of specific clock components and to investigate affected downstream pathways to correlate to changes occurring in patients. This information can provide important insights into the underlying mechanisms of circadian disruption in cancer and inform about the development of potential therapeutic interventions.

2.3.2. The role of circadian clock in neurodegeneration

Several neurodegenerative diseases (72) manifest early symptoms of circadian disturbances such as reflected in sleep/wake cycles in dementia (73), Alzheimer's Disease (AD) (74), and Parkinson's Disease (PD) (75) suggesting that the circadian system's malfunctioning may have a critical and even a causal role in the pathologic development of these disorders (76). Furthermore, there are reports of disrupted circadian oscillations among ND patients and daily variations in symptoms. Patients reported more troublesome motor symptoms in the evening hours (77, 78), particularly in advanced disease stages in both AD and PD. This 'Sundowning Effect' results in range of clinical symptoms including increased cognitive malfunctioning, failure in thermal regulation, or mental breakdowns in patients, all associated with circadian variations. There is an improvement in both motor and non-motor symptoms during morning hours, commonly known as the "sleep-benefit" (79).

PD is anticipated to impact more than 10 million people in the next two decades and is the second most prevalent ND currently (80). It is characterized by both motor symptoms like muscular rigidity, and non-motor symptoms such as tiredness or musculoskeletal pain. Since most PD cases are sporadic and have no known genetic cause, there is no cure or preventive treatment for this disease, which emphasizes the urge to identify novel molecular markers to target the underlying mechanisms. Decreased response to levodopa, a commonly prescribed first line drug for management of PD, in the evening doses compared to morning administration (81); complain of night time akinesia (the loss of voluntary muscle movement) (82) among PD patients suggests a potential role for improvement of disease management by targeting the circadian rhythms to revert some of the disease related symptoms. Also, at the molecular level, GWAS (Genome Wide Association Studies) results revealed an association between single nucleotide polymorphisms of core clock genes and distinct PD phenotypes. These included tremor-dominant phenotype in variants of *ARNTL* (83) (rs900147 variant) ; postural instable phenotype in variants of *PER1* (83) (rs2253820 variant) and postural instable phenotype in variant of *CLOCK* (311T/C variant) (84). Interestingly, the usage of bright light therapy, a dominant Zeitgeber for humans to readjust the circadian system, showed a benefit for management of motor and non-motor symptoms (e.g., sleep behavior) in several neurodegenerative disorders including PD (17, 50, 85, 86).

Particularly in Idiopathic PD (IPD), which describes PD of unknown genetic origin without any mutations in the previously identified risk-associated genes, circadian alterations may provide a benefit to better characterize patient subgroups, diagnose and optimize treatment. Yet, there are currently no available time course-datasets derived from humans in IPD, which we were able to extract and analyze in the scope of this thesis. Therefore, to partially overcome this obstacle, we complemented our computational analysis with a publicly available microarray dataset derived from blood samples of a cohort of IPD patients and identified transcriptomics alterations and compared these to a core-clock KO model of cancer, to identify the common transcriptional signatures occurring upon-perturbation of core-clock genes. Although the results require further validation by controlling the alteration of gene expression in the key genes in humans using circadian assessment methods and time-course datasets, the common alterations observed at mean expression level resemble similar patterns in *in vitro* CRC model and IPD datasets. These findings suggest that these alterations may be translated to future studies at this extent. Using the time-course CRC datasets as a comparison, we wanted to identify circadian alterations at transcriptomics level of our short-listed set of genes coming from cellular pathways, which were differentially regulated in the same way (up/down-regulation) in both diseases. The identified targets might further be explored in context of IPD for its role in pathological development to investigate its potential as a therapeutic or diagnostic marker.

2.4. The research gap and aim of the thesis

The pathological development of cancer and neurodegeneration (ND) governs disruption of common cellular mechanisms. As above-mentioned, these include alterations in DNA repair, redox homeostasis, cell cycle and immune system. At the crossroad of these pathologies, the loss of circadian regulation as a result of disruption of core-clock and Clock Controlled Genes (CCGs) lies. Previous research has focused on identifying links between circadian rhythms (CRs) in connection to cellular mechanisms linking the neurodegeneration and cancer but, to the best of our knowledge, this is the first study investigating the role of disruption of particular core-clock genes in this interplay between both pathologies. In this thesis, we hypothesized that alterations of disease associated cellular pathways correlate with Knock Out (KO) of particular core-clock genes and circadian regulation plays a role in mechanisms linked to cancer and neurodegeneration. To address this hypothesis, we established research objectives that seek to answer the following questions:

1. Does manipulation of core-clock genes (*ARNTL*; *PER2*; *NR1D1*) affect cellular pathways commonly associated with cancer and neurodegeneration?
2. Are alterations in these cellular pathways correlated with changes observed in IPD patients?
3. Do observed gene expression changes in *in vitro* CRC and IPD datasets relate to cancer hallmark networks?
4. Do these common alterations contribute to any clinical characteristics, such as survival rates in CRC patients, for example, in The Cancer Genome Atlas Colon Adenocarcinoma (TCGA-COAD) dataset?

For this purpose, transcriptomics datasets derived from distinct clock phenotypes were chosen to investigate the impact and level of clock disruption in common pathways altered in cancer and neurodegeneration. To do so, RNA-seq datasets (ArrayExpress accession number (no.): E-MTAB-9701) derived from *in-vitro* WT HCT116 *CRC* cells with a functional circadian rhythm and clock KO cells (HCT116-*ARNTL*^{KO}; HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} cells) representing disrupted CRs were analyzed. By complementing this analysis with a microarray dataset (GEO accession no.: GSE99039 (87)) obtained from blood samples of a IPD patients' (N=233) cohort and healthy controls (N=205), we aimed to investigate whether the transcriptomics changes occurring upon KO of core-clock elements resemble those in PD patients in the cellular pathways, which were pre-selected due to their involvement in the overlap of cancer and ND may further be used to identify common targets for development of diagnostic and monitoring tools for these two aging-induced pathologies. The genes involved in cellular pathways related to cancer and ND, thereby our main focus in the scope of this thesis further revealed changes in circadian rhythmicity and mean expression level of candidates upon disruption of core-clock genes. To further demonstrate the effect of these gene expression alterations on the clinical outcomes, we examined the RNA-seq dataset of the colon cancer patients from the publicly available TCGA-COAD cohort (88) which showed a significant impact on the overall survival.

3. Methods

3.1. Generation of time course RNA-seq datasets from HCT116 cells

The subsequent sections (3.1.1, 3.1.2 and 3.1.3) about the maintenance of the cells, generation of the KO cells and preparation of samples for the RNA-seq time course-dataset were performed as provided declaration of author contributions (section 8) by the experimental colleague in the group and further details for the experimental analysis are found (see please reference (89) for further details). In the scope of this thesis, a computational analysis of the mentioned datasets was performed.

3.1.1. Cell culture

HCT116 cell line of human CRC (ATCC CCL-247) and its KOs (*ARNTL*, *PER2*, and *NR1D1*) were grown using a medium composed of Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA), with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. The cells were then incubated at 37°C with 5% CO_2 in a humid environment.

3.1.2. HCT116 cells CRISPR-Cas9 Knockout generation

HCT116-KOs (*ARNTL*, *PER2*, and *NR1D1)* were generated via transfection of HCT116-WT cells with a GFP marker and the FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). CRISPR-Cas9 plasmids containing guide RNAs designed to target several exons of the genes. After 48 h, GFP (+) cells were sorted with an S3e cell sorter (Bio-Rad Laboratories, Hercules, CA, USA) and seeded. The clones were expanded, and their success was confirmed by analyzing via Sanger sequencing.

3.1.3. Preparation of samples for time course RNA-seq

Triplicate seeded HCT116 cells in 12-well plates ($2x10^5$ cells per well) were synchronized via medium exchange and samples were collected between 9 h - 54 h with a 3 h interval post-synchronization. RNA was then isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to check the concentration. The preparation of mRNA libraries was carried out with TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced at the GeneCore Facility of the European Molecular Biology Laboratory (Heidelberg, Germany) using Illumina NextSeq 500 platform, with paired-end reads of 75-bp and an average depth of 100 M. The generated RNA-seq time course dataset (from HCT116-WT and KO cells) was deposited to ArrayExpress (accession no.:

E-MTAB- 9701) and made publicly available upon publication of corresponding papers (please see the references (89-91) for further information).

3.2. Data pre-processing

The raw RNA-seq reads underwent quality control (QC) using FastQC (v0.11.7) (92) and had adapter sequences removed via Trimmomatic (v 0.38) (93). Genome alignment was done with the reference for Homo_sapiens (GRCh38, Ensembl release 92) using the STAR algorithm (version 2.6.0a) (94).Transcript quantification was achieved using Salmon (version 0.10.2) (95) using default settings. The tximport package (v1.6.0) (96) was utilized to standardize the counts of Transcripts per Million (TPM). The scaled TPM counts were then converted to gene-level summarized count estimates; lowly expressed genes were removed and only counts with $mean_{expression\_over\_time\text{-}course} \geq 0.5$ CPM retained. The cpm function was used to retrieve log2-transformed count estimates and renormalized with Trimmed Mean of M-values (TMM) normalization from edgeR (version 3.20.9) (97). For downstream analysis, we obtained TCGA colon adenocarcinoma RNA-seq datasets (denoted as COAD, from platform Illumina HiSeq 2000 available at https://portal.gdc.cancer.gov) and related demographic information using the TCGAbiolinks R package (98). The TCGA-COAD dataset comprised of 285 primary tumor and 41 healthy samples (derived from adjacent tissue). Raw counts were aligned to the reference genome Homo sapiens (GRCh37, hg19) and downloaded from the legacy achieve for downstream analysis. Subsequently, TMM normalization from edgeR package (97) was used and the mean variance relationship of counts was assessed using voom functionality from the limma R package (version 3.14) (99). A low gene expression threshold set (logCPM > 0.5, in at least half of the total samples) and batch correction was performed based on the plate information.

The raw publicly available microarray dataset was retrieved from GEO (accession no.: GSE99039) using the R package GEOquery (100). The blood samples within the dataset were obtained from 205 patients diagnosed with Idiopathic Parkinson's Disease (IPD) and 233 healthy controls. To preprocess the arrays, we utilized the Robust Multi-array Average (RMA) procedure with the R affy package (101) and quality assessment was performed using R arrayQualityMetrics (102). Following QC, 6 arrays that failed 2 or more QC modules assessing the distance between arrays; the distribution via boxplots and MA plots transforming data into log average vs ratios to identify outliers. One control participant with *PINK1* mutation a gene, which is associated with early onset PD (103, 104), were removed to avoid potential bias. As such a total of 204 IPD samples and 227 control samples kept for further downstream analysis. The batch correction of arrays was performed using ComBat R package (105) and annotations were mapped using the hgu133plus2.db package (version 3.2.3) (106). The probe with highest average expression was retained for genes, which were mapped to multiple probes. Based on the histogram of intensity distribution of gene counts, a low expression cutoff (<4) was set.

3.3. Rhythmicity analysis

The RAIN algorithm was utilized to identify circadian (24 hour (h) period) and ultradian (12 h and 8 h period) rhythms on unlogged gene expression values (107). The *p*-values were corrected for multiple testing using the Benjamini-Hochberg (BH) method. The HarmonicRegression (108) (version 1.91) package in R was used to determine oscillatory parameters (amplitudes and phases), where significance was set at $q < 0.05$ and relative amplitude ≥ 0.1. The DODR (Detection of Differential Rhythmicity) (109) method was applied to identify genes that were rhythmically expressed in at least one cell line, and pairwise comparison was conducted between HCT116 WT and each KO. DODR *p*-values were BH adjusted for multiple testing, and a significance threshold of $q < 0.05$ was set.

3.4. Differential expression analysis

To analyze differential gene expression in the IPD microarray dataset, the limma package (version 3.14) in R was used with default parameters (99). Significantly up- and down-regulated genes were identified with a p-value cutoff (99%, $p < 0.01$) and $|\log_2$ fold change (FC)$| \geq 0.1$. A linear model with circadian coefficients was fitted on the mean-variance assessed and normalized counts from the RNA-seq time-series data using the voom function (110). The limorhyde package (version 1.0.1) (111) and limma pipeline were used to compare the knockout cell lines to the HCT116-WT condition, with a significance threshold set to $q < 0.05$ and $|\log_2 FC| > 0.58$. When comparing both RNA-seq and microarray datasets, the unified cutoff ($p < 0.01$ and $|\log_2 FC| \geq 0.1$) was used. In the TCGA-COAD RNA-seq dataset, differential expression analysis was performed using the limma package (99), with genes that were significantly differentially expressed between cancer versus normal samples determined based on a $|\log_2 FC| > 1$ and BH-adjusted $q < 0.01$.

3.5. Aggregation of cancer and neurodegeneration KEGG Pathways

Prior to downstream analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with cancer and neurodegeneration were chosen for analysis. These included all KEGG human pathways for "cell growth and death" from the cellular processes module, "replication and repair" pathways from the genetic information and processing module and pathways from the "immune system" module (112). As there were no redox homeostasis pathways explicitly listed in KEGG, we also incorporated "transport and catabolism" and "energy metabolism" pathways from the cellular processes' module. Additionally, the PD pathway (hsa05012) under the KEGG human disease module was included and we carried out a literature search to extend this list with other PD-associated genes. The KEGGREST (v1.30.1) R package (113) was utilized to obtain the lists from KEGG. After eliminating duplicated genes, a list of 2507 unique genes were generated.

3.6. Demographics information for the cohort of IPD patients

The IPD patient's cohort was originally published (87), and all patients were evaluated clinically at referring centers rigorously. The patients without an observable dopaminergic deficiency in tomography scans were excluded. The demographic information on the patient and control groups (see please references (87) and Yalçin, M., Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Table 1**, (91)).

3.7. Overall survival analysis

The differentially expressed genes (totalling 3234) were subjected to Kaplan-Meier (KM) survival analysis to identify genes that had a significant association with overall survival in TCGA-COAD cohort. From this analysis, 289 genes were identified to be significantly associated with survival ($q < 0.05$). For cancer samples, the overall survival was evaluated using the date of last follow-up or days until death. To compare the change in gene expression levels, each expression value was classified into high and low expression subgroups based on the median gene expression level. The KM analysis was utilized with the 'survival' package (version 3.2.12) in R and survminer package (version 0.4.9) to create visual representations of the data. Potential influence of additional clinical factors such as age, sex, and tumor stage on results were assessed, and Cox regression model was used to stratify the outcomes based on stage.

4. Results

4.1. The rhythmic gene expression of cancer- and neurodegeneration-related pathways is altered due to the perturbation of core-clock genes

Cancer and neurodegeneration (ND) are two highly prevalent pathologies that share a number of common disrupted cellular processes, including alterations as above-mentioned in the DNA repair and cell cycle, redox homeostasis and the immune system. Recent studies suggest that alterations in the expression of certain elements of these pathways, which are controlled by the circadian clock, may be related to these diseases. To investigate this, here we carried out a computational analysis of microarray datasets (438 samples) taken from whole blood of patients with IPD and time series RNA-seq datasets (64 samples) from CRC cell lines that were designed to mimic disruptions of different core-clock genes (*ARNTL*, *PER2* or *NR1D1*).

The data for IPD patients was obtained from whole blood samples collected from 205 patients, and 233 controls (accession no.: GSE99039 (87)). The CRC cell line data was newly generated for HCT116-WT and HCT116-*ARNTL*^{KO}; HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} cells (accession no.: E-MTAB-9701 (89, 91)), in addition to previously generated data from CRC cells (SW480, SW620 cells) derived from the primary tumor and metastatic site of the same individual (accession no: E-MTAB-7779 (114)).To better understand the rhythmic expression alterations in cellular pathways involved in both cancer and ND, we conducted a rhythmicity analysis on core-clock KO CRC time-course RNA-seq data. We identified genes that oscillated with circadian (24 h) and ultradian (8-12 h) periods and used a differential expression analysis to explore the mean level changes in HCT116 cells and IPD datasets. We curated a list of 2507 unique genes (see please published version **refer to Table S1-S10 in the publication** (91)), denoted as 'genes of interest' hereafter, including those from commonly disrupted pathways in both cancer and ND, such as the immune system, DNA damage, cell cycle, and redox homeostasis, as well as the core-clock network (CCN) and extended-CCN (ECCN) (115, 116). In our study, we found that the HCT116 KO cells exhibited a decrease in the overall number of rhythmic genes with respect to the WT cells, for both circadian and ultradian periods (**Figure 4A-D**). Furthermore, the distribution of peak expression time (phase) for total rhythmic genes in the transcriptome and genes of interest showed changes. KO of *ARNTL* (**Figure 4B**) and *PER2* (**Figure 4C**) showed more prominent changes in number of rhythmic genes, indicating that the perturbation of specific core-clock elements might affect the expression of particular downstream pathways differently.

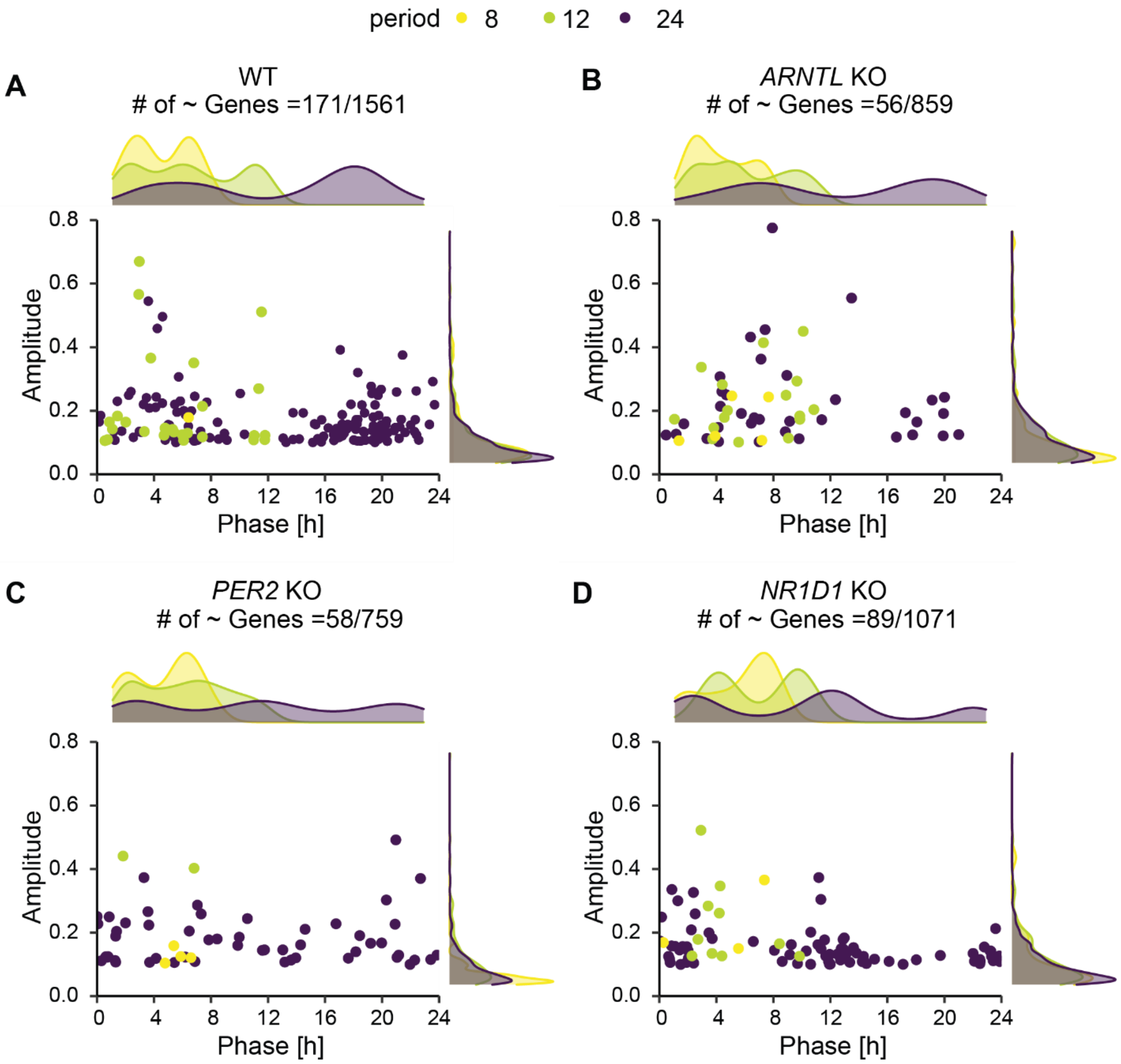

Figure 4. Upon KO of core-clock genes rhythmic expression of genes of interest is altered. Global number of significantly oscillating genes and genes of interest are indicated above each panel. Pre-selected genes of interest are visualized in the scatter plots whereas the density plots refer to global distribution of oscillatory properties (phase and amplitude) for tested periods for ultradian (8 h in yellow, and 12 h in green) and for circadian (24 h in purple) genes in **(A)** HCT116-WT cells **(B)** HCT116-*ARNTL*^{KO} **(C)** HCT116-*PER2*^{KO} **(D)** HCT116-*NR1D1* ^{KO} cells. (**Figure modified from <u>Yalçin, M.</u>,** Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 2 in the publication** (91)).

4.2. Differentially regulated genes in cellular pathways shares an overlap between CRC and IPD datasets and the co-expression of core clock network varies in IPD patients

To gain a better understanding of the correlation between changes in gene expression in IPD dataset and our core-clock disrupted CRC model, we conducted an analysis of differential expression in each dataset. To further investigate potential sex-specific differences, we also divided the IPD data into subgroups for males and females and carried out a functional enrichment analysis of global differentially expressed genes list revealing distinct sets of genes of interest in each subgroup. Following our differential expression analysis, we identified overlapping and unique set of genes of interest in each clock KO and the IPD cohort. Our analysis revealed that HCT116-*PER2*^{KO} cells exhibited the most significant alteration in a synergistic manner (i.e., both up-/downregulated) in the expression levels of the genes of interest compared to the IPD cohort followed by HCT116-*NR1D1*^{KO}; HCT116-*ARNTL*^{KO} cells (**Figure 5**).

Our comparative analysis revealed nine genes that exhibited altered expression in all knockouts and in IPD patient samples. These included axonal guidance and structure related gene *Vasodilator Stimulated Phosphoprotein* (*VASP*)*,* immunity related genes *Insulin Like Growth Factor 2 Receptor (IGF2R), Serpin Family A Member 1 (SERPINA1), Histone Deacetylase 2 (HDAC2), Transforming Growth Factor Beta 1 (TGFB);* cell cycle and proliferation genes *Cyclin Dependent Kinase Inhibitor 1C* (*CDKN1C)* and *Sp1 Transcription Factor* (*SP1)*; oxidase activity and endocytosis associated *Cytochrome B-245 Alpha Chain* (*CYBA*) and *Low Density Lipoprotein Receptor Adaptor Protein 1* (*LDLRAP1*) (**Figure 5**). After subgrouping the IPD cohort by sex, we conducted a gene expression analysis in both females (90 IPD vs 142 controls) and males (101 IPD vs 70 controls), which revealed a smaller number of genes of interest that showed differential expression in females compared to males. Although there was a greater overlap in the number of differentially expressed genes of interest between males and core-clock CRC KOs, observed changes were in the opposite direction (up-/down regulation) between these datasets **refer to Figure S8 in the publication** (91)).

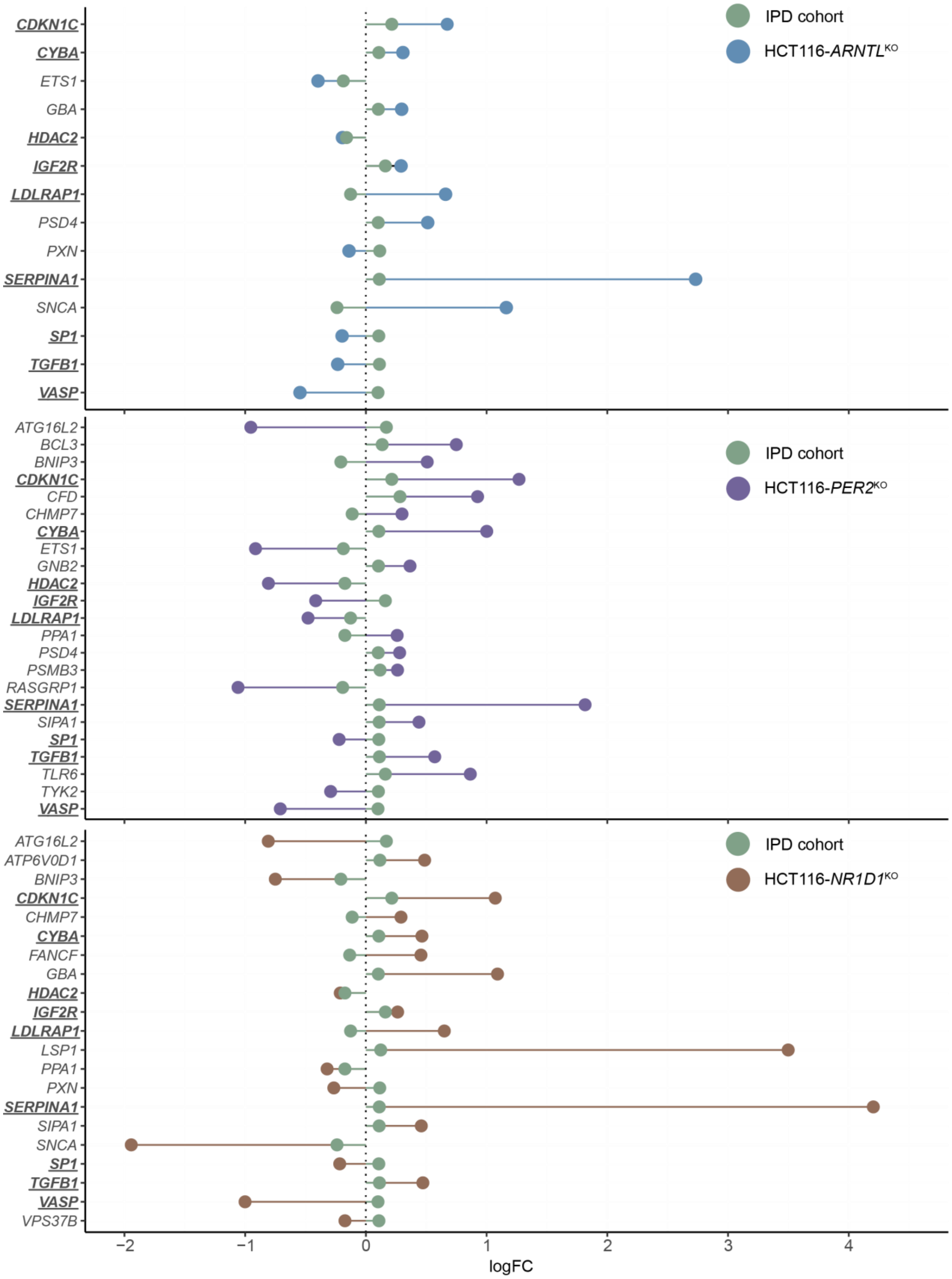

Figure 5. Common genes of interest differentially expressed in HCT116-KO cells and IPD cohort. The direction of changes (up or down-regulation) at mean gene expression levels were reflected with lollipop plots for the genes of interest shared between HCT116-*ARNTL*^{KO}, HCT116-*PER2*^{KO}, HCT116-*NR1D1*^{KO} and IPD cohort versus respective controls. **(Figure modified from <u>Yalçin, M.</u>,** Malhan, D., Basti, A.,

Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 4 in the publication** (91)).

Our investigation also showed that *D-Box Binding PAR BZIP Transcription Factor* (*DBP*), a central regulator of the circadian clock (117), exhibited differential expression in the HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} cells, as well as in female IPD patients. We subsequently identified the top 100 differentially expressed genes in both subgroups, which did not reveal a distinctive gene expression profile that could clearly differentiate IPD patients from controls. However, we observed association of a unique set of signatures out of differentially expressed genes of interest in females and males. We did not detect any differential expression of Core Clock Network (CCN) and Extended-CCN (ECCN) elements in the overall cohort and in sub-group analysis (based on sex) **refer to Figure S9-11 in the publication** (91). To reveal potential co-expression changes we conducted a correlation analysis with the IPD dataset. The correlation analysis between the CCN (**Figure 6**) elements in male and female IPD subgroups showed weaker correlation with respect to corresponding control groups, indicating that the association between these genes is not as robust in IPD patients.

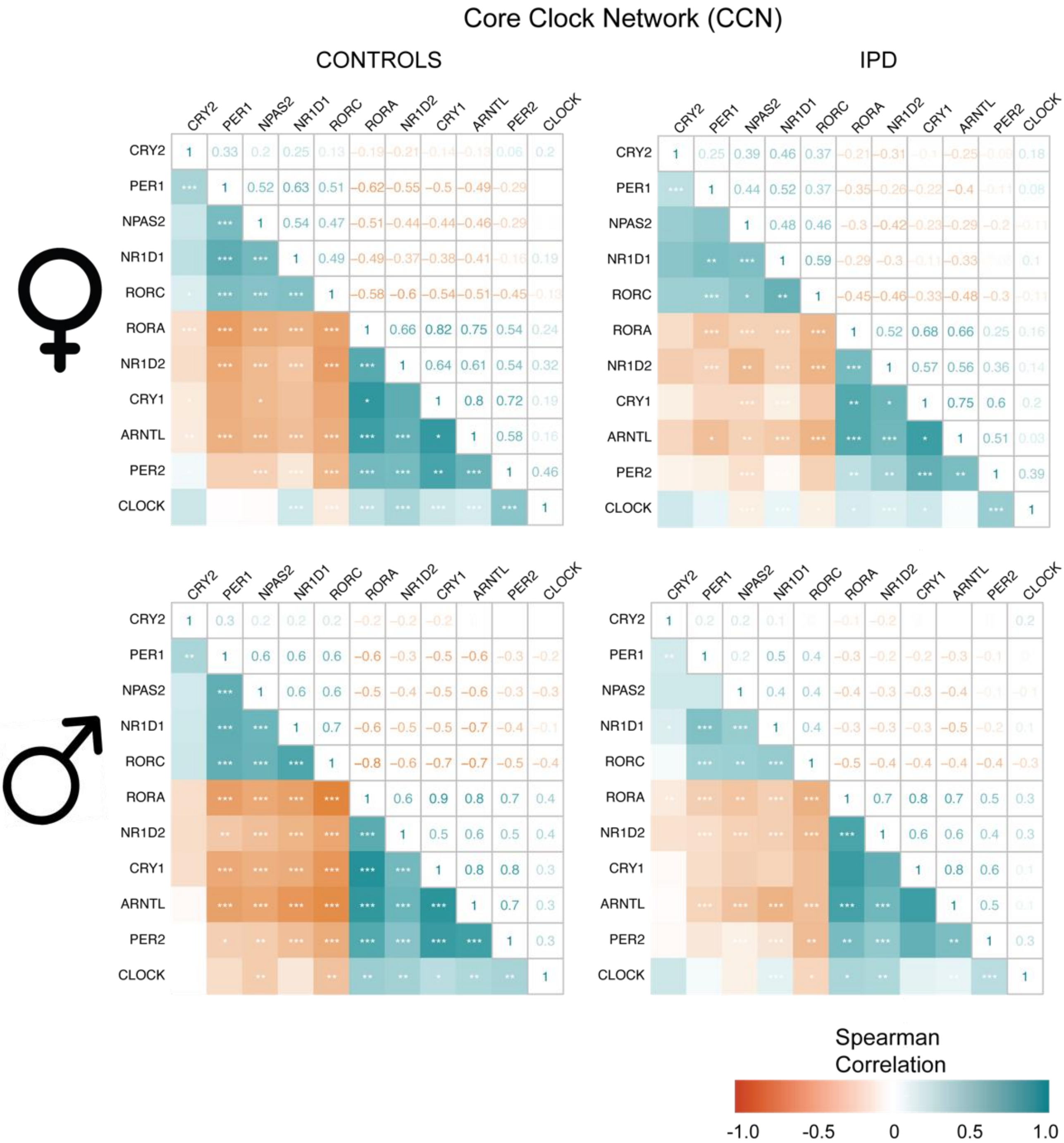

Figure 6. Correlation of CCN genes is altered in IPD patients versus controls (sex separated). Correlation heatmaps reflect Spearman coefficient values in females and males. Statistical significance is denoted as (*) : q<0.05 ; (**) : q<0.01 ; (***) : q<0.001. **(Figure modified from Yalçin, M.,** Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 6 in the publication** (91) **for integration of statistical representations)**.

4.3. Disruption of core-clock genes leads to rhythmicity alterations in commonly differentially regulated genes of CRC cells and IPD cohort

To evaluate how clock disruption affects the oscillatory patterns of the top differentially regulated genes in the IPD cohort, we identified the genes from commonly disrupted cellular pathways, from the list of the top 100 differentially expressed (DE) gene set in the full cohort, as well as in males and females. Due to single time point collection of the IPD dataset and lack of available information on sampling time, we used CRC cell time course data to examine changes in circadian gene expression in this section. To further illustrate the impact of core-clock alteration on the rhythmicity properties of the genes of interest, such as the loss or gain of rhythmicity or changes in amplitude (the distance to the mean level of the peak to through of an oscillation) or phases (peak expression time), we conducted a differential rhythmicity analysis for all rhythmic genes of interest.

Among IPD female patients top 100 DE gene set, we found *Glucosylceramidase Beta 1* (*GBA*), a lysosomal processing regulator functioning was circadian expressed in HCT116WT cells **refer to Figure S13 in the publication** (91)). *GBA* is a common gene identified as a risk factor in IPD patients whose mutation is associated with increased susceptibility (118). Perturbation of core-clock genes led to the loss of circadian rhythmicity in *GBA* across all HCT116-KO cells (**Figure 7**). In males, *Cytochrome B-245 Alpha Chain* (*CYBA),* which is involved in regulation of oxidative stress, immunity and phagocytosis, was differentially expressed **refer to Figure S13 in the publication** (91)). Circadian expression of *CYBA* in HCT116^{WT} cells was abolished upon perturbation of core-clock genes. Notably, we observed that *Dopamine Receptor D1* (*DRD1)* encoding the dopamine receptor that is expressed in high quantities within the central nervous system showed an alteration in its rhythmicity upon perturbation of all core-clock genes (**Figure 7**). Among other notable PD-associated genes that exhibit an alteration in their circadian profile *ADRM1 26S Proteasome Ubiquitin Receptor* (*ADRM1),* involved in regulation of ubiquitination and *Tubulin Beta 2A Class IIa* (*TUBB2A)* a regulator of neuronal communication and intracellular transport were differentially expressed in female and male IPD patients respectively and showed a loss of circadian rhythmicity in all KO cells (**Figure 7**). Taken together, our findings provide a foundation for contribution of core-clock disruption to shared gene expression alterations between cancer and neurodegeneration.

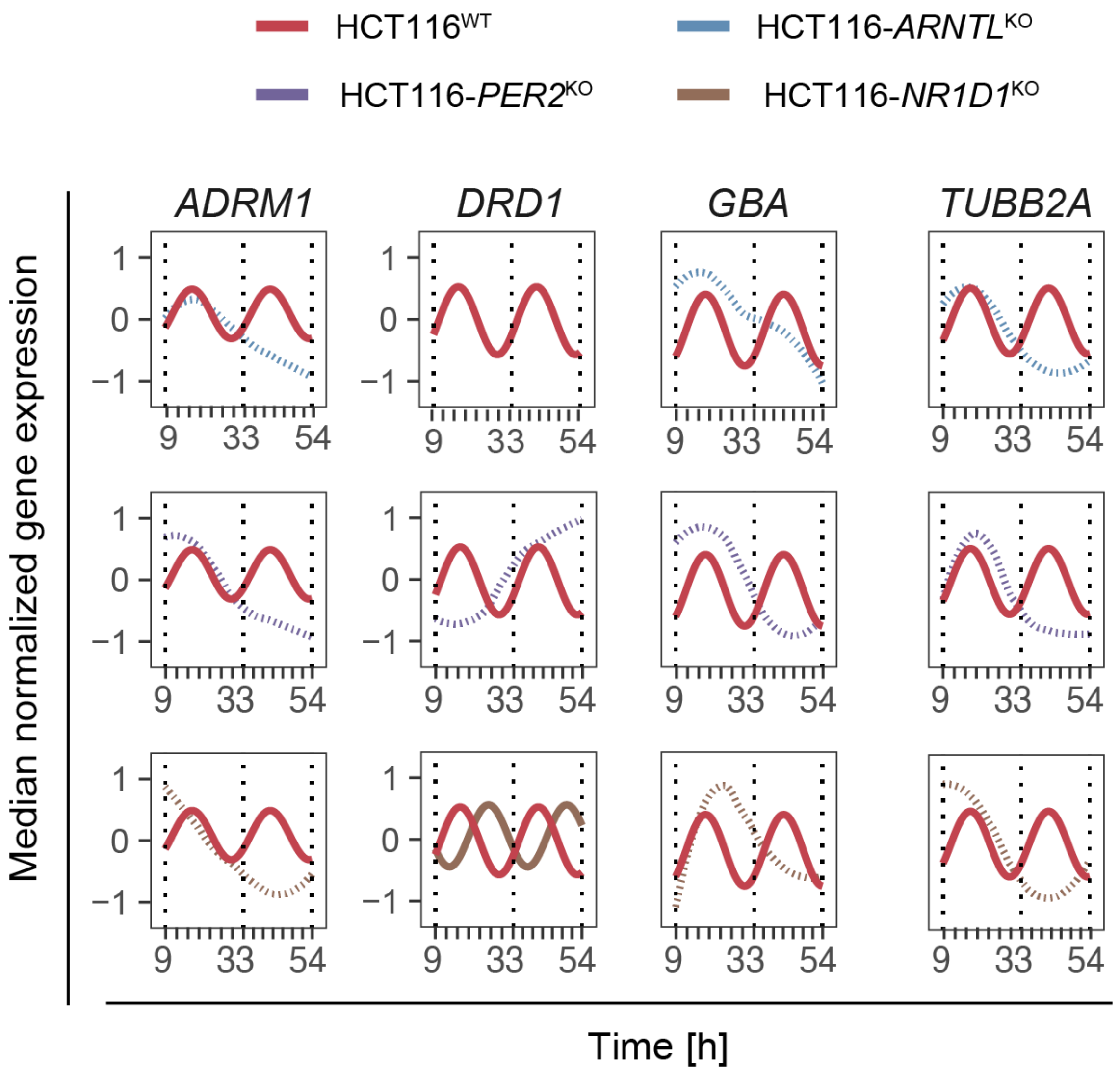

Figure 7. Circadian expression profiles of short-listed PD-associated genes in HCT116^{WT} cells and HCT116-*ARNTL*^{KO} (top row) in HCT116^{WT} and HCT116-*PER2*^{KO} (middle row) in HCT116^{WT} and HCT116-*NR1D1*^{KO} (bottom row) cells. **(Figure modified from <u>Yalçin, M.</u>,** Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 7 in the publication** (91))

4.4. Differentially regulated genes of interest are connected to cancer hallmark pathways and can affect survival of CRC Patients

We examined the correlation of our findings with those genes known to be associated with cancer in the Cancer Hallmark Genes (CHG) database (119). We selected genes based on differential rhythmicity (for genes showing significant results in at least one of the KO cells with respect to WT), common differentially expressed genes in HCT116^{KO} cells and the IPD cohort, and the top differentially regulated genes (100 differentially expressed genes that showed significant differences) within the IPD dataset. About 40% of the differentially regulated genes were linked to cancer hallmarks, with the highest proportion being associated with signatures from "sustaining proliferative signalling", "resistance to cell death", and "activation of invasion and metastasis" (**Figure 8**).

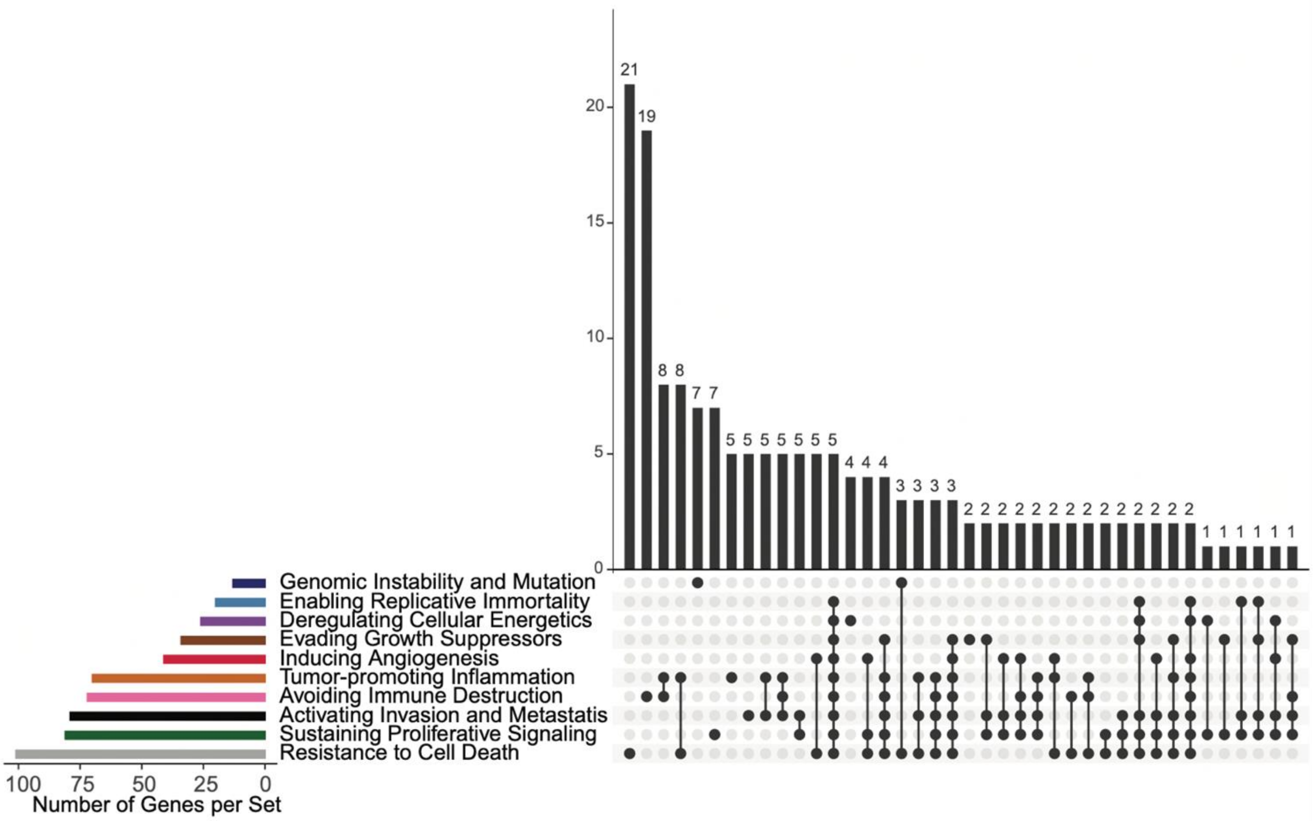

Figure 8. Association of differentially regulated genes of interest to cancer hallmark gene networks. The upset plots reflect the number of genes related to each hallmark (color coded bar plot). The number of unique gene sets associated to one or more cancer hallmarks (connected lines) are reflected in dark grey. **(Figure modified from <u>Yalçin, M.</u>,** Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 8A in the publication** (91)).

To explore the association between the dysregulated genes of interest in IPD and cancer datasets with patient survival, we conducted a Kaplan Meier survival analysis using the TCGA-COAD dataset. Our analysis identified 289 genes detected, to be significantly associated with overall survival among DE genes between cancer versus normal tissue samples. We examined various clinical variables, and age at diagnosis and sex were not significantly related to survival **refer to Figure S14 in the publication** (91)). Nevertheless, the stage showed a significant association with survival, which therefore was used as a stratification factor using Cox-models. We observed that *P21 (RAC) Activated Kinase 6* (*PAK6*), linked to 'evading growth suppressors', 'cell death resistance', 'sustaining proliferative signalling', 'immune destruction' and 'activation of invasion and metastasis', along with *Solute Carrier Family 11 Member 1* (*SLC11A1*), associated with 'resistance to cell death', and *Tubulin Beta 6 Class V* (*TUBB6*), associated with 'tumor promoting inflammation' and 'activation of invasion and metastasis', significantly affected survival when considering stage stratification (**Figure 9**). The *TUBB6* downregulation was associated with increased survival probability in the TCGA-COAD dataset which showed altered circadian expression in KO cells. Downregulation of *PAK6*, *SLC11A1, TUBB6* which were differentially regulated in HCT116 KO cells and in IPD patients, resulted in higher survival probability when downregulated (**Figure 9**). Taken together our results pointed to several genes, that show altered expression in both CRC cells and IPD patients, are associated with cancer hallmark networks and significantly influence survival outcomes in patients with colon cancer.

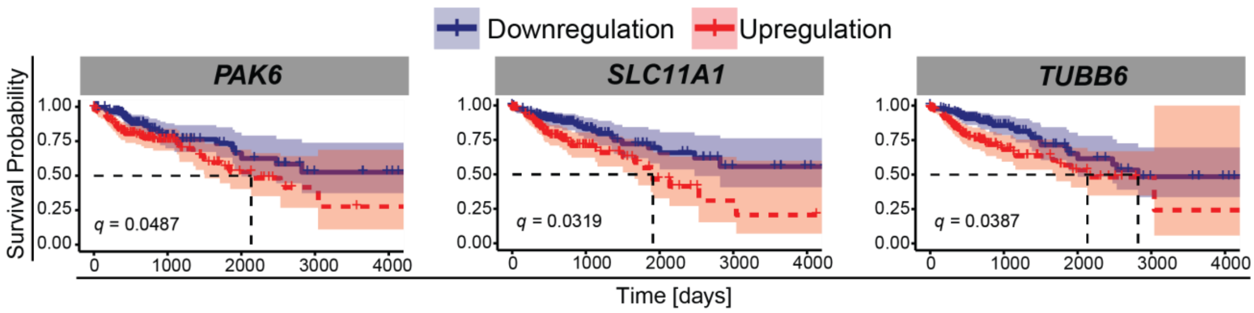

Figure 9. Commonly differentially regulated genes in CRC cells and IPD patients reflect a role in overall survival of colon adenocarcinoma patients. Gene expression of TCGA-COAD dataset was assigned into high and low groups (based on the median) and a comparison of survival time (in days) was performed. **(Figure modified from <u>Yalçin, M.</u>,** Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Figure 8B in the publication** (91)).

5. Discussion

In the intersection of cancer and neurodegeneration, lies the loss of circadian regulation, which occurs as a result of the disruption of core-clock genes and Clock Controlled Genes. Although the cellular outcomes underlying these pathological processes appear to be opposite (31, 33) both Parkinson's disease and cancer are age-related that share accumulation of erroneous events that alter common downstream cellular pathways. While prior research has examined the connection between the clock and cellular mechanisms that link neurodegeneration or cancer, our study represents the first investigation into the impact of disruption of core-clock genes in this association. We provide evidence suggesting that genes involved in commonly disrupted cellular pathways in cancer and ND including maintaining redox balance, regulating the immune system, and controlling cell cycle and DNA repair, are likely regulated by the circadian clock.

5.1. Interpretation and embedding of the results into the research context

In this study, we aimed to elucidate the impact of core-clock genes on differential regulation of cellular pathways associated with cancer and ND at the transcriptomics level. We discovered that disturbing the core-clock genes had an impact on the rhythmicity of genes involved in cancer and ND. Our research indicates that *PER2*, which was previously recognized for its tumor suppressor activity (120-122), was responsible for a significant decrease in the number of oscillating genes (with all periods) and for the most significant change in phase distribution across all periods when it was knocked out, compared to WT cells. Our research revealed that several clock genes such as, *CRY1*, *DBP*, *NR1D1* showed differential rhythmicity in all KO cells. Moreover, some of the genes exhibiting differential rhythmicity in the CRC data were also differentially regulated in the IPD cohort according to the results at mean gene expression level. *DBP,* which was differentially expressed exclusively in the female subgroup, lost its circadian activity in HCT116-*ARNTL*^{KO} cells and was upregulated in HCT116-*NR1D1*^{KO} and HCT116-*PER2*^{KO} cells, pointing to a sex-specific variation in gene expression. Existence of more genes of interest with differential expression changes in the synergistic way in the female subgroup as in the clock KO cells compared to the male subgroup further supported this. Previously, the circadian activity of *DBP* has been shown to be abolished in *ARNTL*^{KO} mice and linked to neurodegeneration (123). Additionally, the circadian clock could play a crucial role in the relationship between healthy and cancerous tissues. A recent study found a decrease in the circadian activity of *Dbp* in healthy mice with respect to those with colon cancer (124) underscoring the role of the circadian clock in the pathways commonly altered between these pathologies. Although no other clock gene was differentially expressed in the IPD patient cohort, our data indicated weaker correlation of Core-Clock Network (CCN) and Extended-CCN elements in IPD patients in female and male patients with respect to controls. This suggests a shift in the relative expression of clock genes likely because of PD, as previous studies have already reported alterations in the expression of clock genes in PD (125-127). The *DRD1* receptor was found to exhibit a phase shift, particularly a delay in HCT116-*NR1D1*^{KO} cells compared to WT cells, suggesting that circadian factors might play a role in the connection between dopamine receptors and abnormal interactions causing symptoms such as L-dopa-induced dyskinesia.

GBA encodes for a lysosomal enzyme and is a key risk factor for IPD. It was upregulated in HCT116-*ARNTL*^{KO} and HCT116-*NR1D1*^{KO} cells, as well as in IPD patients. In contrast, *SNCA*, a gene that is associated with the aggregation of alpha-synuclein protein in the brain (128), was upregulated only in HCT116-*ARNTL*^{KO} cells, but downregulated in *NR1D1*^{KO} cells and IPD patients. These findings suggest a possible link between circadian dysfunction and fluctuations in motor or non-motor symptoms observed in PD patients with *GBA* or *SNCA* alteration. In addition, perturbations of core-clock genes may yield differential outcomes in gene expression levels (up-/down-regulation), which may affect both cancer and ND in a distinct, core-clock specific manner as pointed by our analysis.

To examine the role of altered genes identified from our analysis with CRC and IPD datasets in the clinical traits, a survival analysis was conducted with TCGA-COAD dataset in which genes such as *TUBB6* and *PAK6* were found to have a significant influence on survival and were also part of cancer hallmark networks. Even though our analysis considered tumor stage, sex and age as potential adjustment factors for stratification, other clinical traits like treatment regime or time may contribute to the prognostic potential of these genes and thus should be further studied. Downregulation of *PAK6* has been linked to higher survival probability in colon cancer patients before and found associated with chemotherapeutic resistance (129). It also interacts with *Leucine-rich repeat kinase 2* (*LRRK2*) (130), whose mutational alteration is associated with a genetic PD form (131), and was upregulated in HCT116-*ARNTL*^{KO}, HCT116-*NR1D1*^{KO} cells, and IPD female patients, suggesting another potential core-clock link between cancer and ND. Similarly, *TUBB6* downregulation was found to yield a better survival probability, which was anticipated as a prognostic marker in various cancer forms previously including ovarian (132), gastric (133) and prostate cancer (134). *TUBB6* is involved in inflammatory-induced cell death response and a direct interactor of *LRRK2 (135, 136)*. Taken together our study suggests that several of the genes identified from the pre-selected cellular pathways are relevant in a CRC and IPD context and are likely regulated by the circadian clock. Moreover, some of these genes influence the clinical traits such as survival in cancer patients, highlighting emerging new associations between cancer and ND with the involvement of the circadian clock (**Figure 10**).

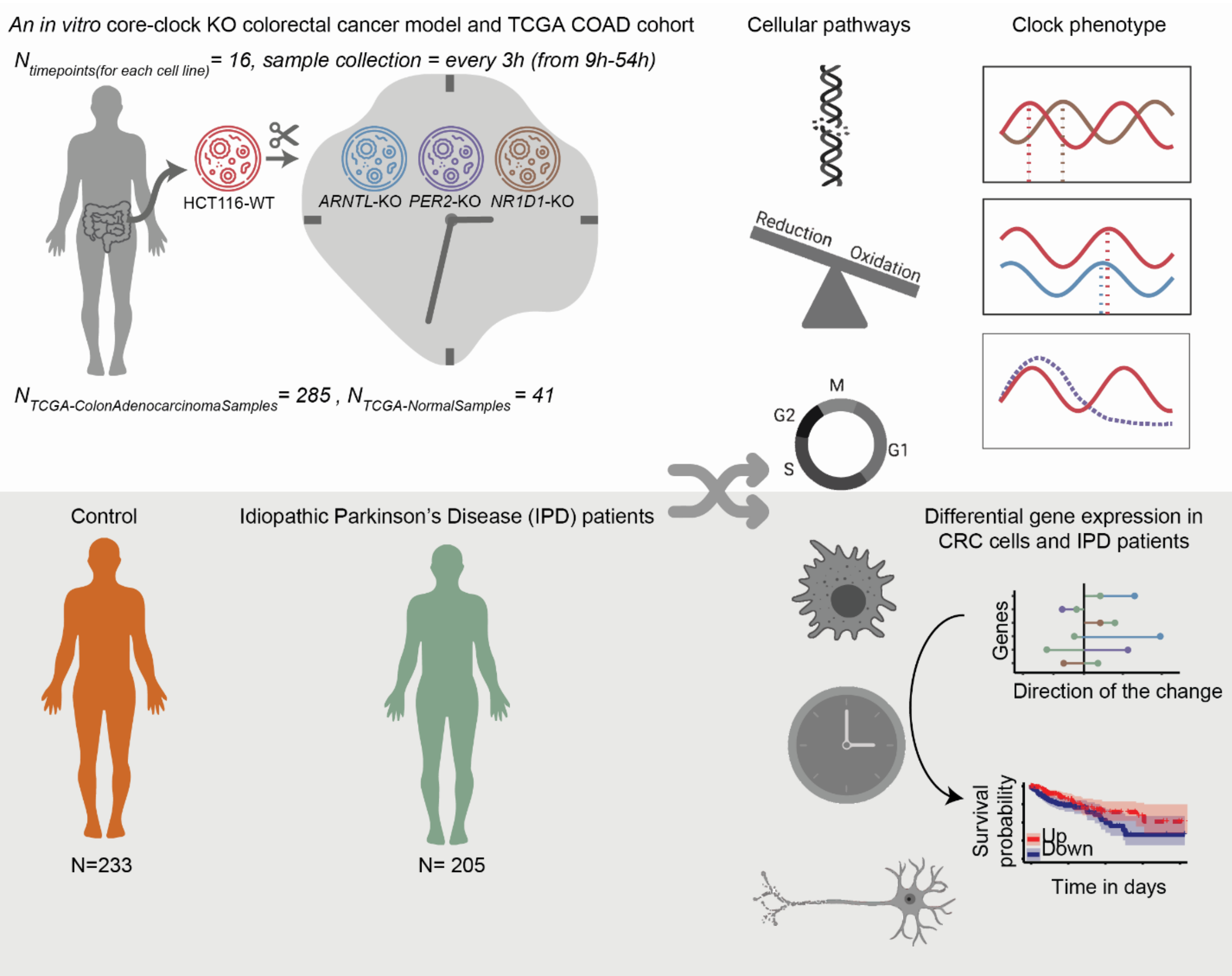

Figure 10. Schematic overview of computational analysis pipeline and major findings. CRC datasets derived from newly generated core-clock KO cells, and publicly available datasets from TCGA (COAD cohort) and a cohort of IPD patients (87) and controls were analyzed. HCT116^{WT} cells were KO using CRISPR/Cas9 methodology targeting *ARNTL*, *PER2* and *NR1D1*, and sequenced with RNA-seq (accession no: E-MTAB-9701). In parallel, a computational analysis was carried out for IPD datasets (accession no: GSE99039). A survival analysis was then performed for the short-listed genes based on the changes in the both datasets and validated in TCGA-COAD dataset (TCGA-COAD HiSeq 2000, available at https://portal.gdc.cancer.gov). (**Figure modified from Yalçin, M.,** Malhan, D., Basti, A., Peralta, A. R.,

Ferreira, J. J., & Relógio, A. (2021). 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, **refer to Graphical Abstract in the publication** (91)).

5.2. Limitations of the study

The internal validity of our study was based on the careful design of our study methodology. Despite the lack of biological replicates in our *in vitro* CRC model (RNA-seq time series data), we were able to circumvent this limitation by collecting data over two circadian cycles, which increases the robustness of the data and can be considered as biological replicates. This design was also found valid as gold standard for circadian gene expression studies (137). Although *in vivo* experimental data in mice could have further strengthened the study, we did not work with animal models in the scope of this thesis. The translation of the results from nocturnal mice to diurnal human patients also requires additional adjustments and may be limited (138, 139). Thus, we intend to validate changes in the circadian expression profile of candidate genes from the pre-selected pathways identified in this thesis in future human cancer studies (140).

The generalizability of our study results may be limited to the population of IPD patients that we studied, and it is possible that our results may not apply to other populations with different genetic backgrounds or clinical characteristics. However, our study methodology was based on rigorous statistical analyses and differential expression analyses, which allowed us to make confident conclusions about the gene expression profiles in the IPD cohort that we studied. Although brain is the primary affected area in context of PD, blood datasets are particularly useful due to easy accessibility compared to brain tissue. Additionally, studies have shown that changes in gene expression patterns can be detected in blood samples of individuals with PD, providing a valuable tool for disease diagnosis and monitoring (141-143). Yet, as neither the timing of blood sample collection nor a time series dataset was available for IPD dataset, a circadian analysis could not be performed. Although computational approaches such as ZeitZeiger (144) or CYCLOPS (145) are available to model the high-dimensional datasets to predict a periodic variable such as time of the day, due to lack of available date and time information for samples, we could not use the IPD cohort as a training dataset to predict such features. Nonetheless, we conducted a differential expression analysis in both CRC and IPD datasets to identify changes in the mean expression levels. Furthermore, we examined the rhythmic expression of pre-selected genes of interest resulting from the IPD datasets in our time-course CRC data. Despite these limitations, our study results provide valuable insights into the circadian regulation of cancer and neurodegeneration-associated pathways and delivers valuable insights for future research into the molecular signatures underlying these pathologies.

5.3. Conclusion and perspectives

The molecular crosstalk between circadian dysfunction and mechanisms underlying cancer and neurodegeneration are just starting to be revealed. Our results shed light on the similarities and differences in the transcriptional changes of genes associated with both pathologies, which lead to distinct outcomes when specific core-clock genes are perturbed. The observed changes in cellular pathways may have prognostic implications for clinical outcomes such as overall survival of colon cancer patients. Taken together findings of this study highlight a role for the circadian regulation of the molecular pathways associated with cancer and ND in IPD patients and core-clock KO CRC cells. Additionally, further research is necessary to investigate the role of circadian regulation in different cancer types and forms of PD. In the future, generation of time-series data in context of such pathologies would allow to identify molecular signatures for prognostic and diagnostic purposes, while considering the circadian profile of disease-associated genes. It is worth noting that patient stratification based on factors, such as sex and stage, may influence the study outcome as shown by our results. The patients' circadian rhythm is a relevant, but often neglected trait that should be considered in future studies, imposing the development of innovative non-invasive approaches for its accurate assessment to bridge the gap between the number of circadian studies and clinical trials to promote arrival of circadian medicine into the clinics.

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7. Statutory Declaration

I, Müge Yalçın, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "“A comprehensive bioinformatic analysis of circadian regulation of cellular pathways in an in-vitro model of colorectal cancer (CRC) and neurodegeneration - Eine umfassende bioinformatische Analyse der zirkadianen Regulierung zellulärer Signalwege in einem in-vitro-Modell für Kolorektalkarzinom (CRC) und Neurodegeneration”, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts, which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility. Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors, http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

8. Declaration of your own contribution to the publications

Müge Yalçin contributed the following to the below listed publications:

Publication #1: Yalçin, M. (**M.Y.**), Malhan, D. (D.M.), Basti, A. (A.B.), Peralta, R. (A.R.P.), Ferreira, J. (J.J.F.), Relógio, A. (A.R.) (2021). 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.

Contribution in detail:

Conceptualization of the study: A.R.;

Computational analysis: M.Y. (quality controls and data pre-processing GSE99039, and TCGA-COAD HiSeq, and normalization (for GSE99039, E-MTAB-9701, E-MTAB-7779 and TCGA-COAD), statistical analysis, rhymicity analysis, differential expression analysis, curation of KEGG pathways, correlation analysis (formation of the correlation matrix) and functional enrichment analysis, survival analysis yielding Figure 1,2,3,4,5 and Figure 7,8, and all supplementary materials, D.M. (quality controls and data pre-processing (E-MTAB-9701) and correlation analysis (visualization) yielding Figure 6,

Experimental analysis: A.B. (generation of the HCT116 KOs, maintenance of the cells and preparation of the libraries for the time-course RNA-seq data (E-MTAB-9701);

Investigation and interpretation of the results: M.Y., D.M., A.B., A.R.P., J.J.F., A.R.

Writing—original draft preparation: M.Y., A.R.

Writing—review and editing: M.Y., D.M., A.B., A.R.P., J.J.F., A.R.

Supervision: J.J.F., A.R.

Funding acquisition: M.Y., A.R.

Publication #2: Yalçin, M. (M.Y.), Mundorf, A. (A.M.), Amatriain-Fernández, S. (S.A.F.), Kalthoff, I. (I.K.), Beucke, J. (J.C.B.), Budde, H. (H.B.), Garthus-Niegel, S. (S.G.N.), Peterburs, J. (J.P.), Relógio, A. (A.R.) (2022). It's about time: the circadian network as a time-keeper for cognitive functioning, locomotor activity, and mental health. Frontiers in Physiology.

Conceptualization: J.C.B, H.B., S.G.N., J.P., A.R.

Computational analysis: M.Y.

Visualization: M.Y. yielding Figure 1,2,3, A.M. yielding Figure 4

Writing—original draft preparation: M.Y. (Introduction, Section: ‘*Making biological time: the molecular and neural circuits mediating generation and functioning of circadian rhythms*’), A.M. (Section: ‘*Circadian regulation of behaviour and cognitive functioning*’), F.T. (Section: ‘*Epidemiological evidence towards circadian regulation of mental health*’), S.A.F (Section: ‘*Circadian rhythms, physical exercise and cognitive processes*’), I.K. (Section: ‘*Circadian regulation of behaviour and cognitive functioning*’), J.C.B. (Section: ‘*Circadian regulation of behaviour and cognitive functioning*’), H.B. (Section: ‘*Circadian rhythms, physical exercise and cognitive processes*’), S.G.N. (Section: ‘*Epidemiological evidence towards circadian regulation of mental health*’), J.P. (Section: ‘*Circadian regulation of behaviour and cognitive functioning*’), A.R. (Introduction, Section: ‘*Making biological time: the molecular and neural circuits mediating generation and functioning of circadian rhythms*’)

Writing reviewing and editing: M.Y., A.M., F.T., S.A.F., I.K., J.C.B., H.B., S.G.N., J.P., A.R.

Supervision: J.C.B, H.B., S.G.N., J.P., A.R.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

9. Printing copy of publications

9.1.1. Printing copy of the publication for Yalçin, M., Malhan, D., Basti, A., Peralta, A. R., Ferreira, J. J., & Relógio, A. (2021). 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

cancers

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Article

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

Müge Yalçin [1,2], Deeksha Malhan [1,2,3], Alireza Basti [1,2,3], Ana Rita Peralta [4,5,6,7], Joaquim J. Ferreira [7,8,9] and Angela Relógio [1,2,3,*]

[1] Institute for Theoretical Biology (ITB), Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, 10117 Berlin, Germany; muege.yalcin@charite.de (M.Y.); deeksha.malhan@charite.de (D.M.); alireza.basti@charite.de (A.B.)
[2] Molecular Cancer Research Center (MKFZ), Medical Department of Hematology, Oncology, and Tumour Immunology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, 10117 Berlin, Germany
[3] Institute for Systems Medicine and Faculty of Human Medicine, MSH Medical School Hamburg, 20457 Hamburg, Germany
[4] EEG/Sleep Laboratory, Department Neurosciences and Mental Health, Hospital de Santa Maria—CHULN, 1649-035 Lisbon, Portugal; anaritaperalta@gmail.com
[5] Department of Neurology, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisbon, Portugal
[6] Instituto de Fisiologia, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisbon, Portugal
[7] CNS-Campus Neurológico Senior, 2560-280 Torres Vedras, Portugal; jferreira@medicina.ulisboa.pt
[8] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisbon, Portugal
[9] Laboratory of Clinical Pharmacology and Therapeutics, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisbon, Portugal
* Correspondence: angela.relogio@charite.de or angela.relogio@medicalschool-hamburg.de

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Simple Summary: Cancer and neurodegenerative diseases are two aging-related pathologies with differential developmental characteristics, but they share altered cellular pathways. Interestingly, dysregulations in the biological clock are reported in both diseases, though the extent and potential consequences of such disruption have not been fully elucidated. In this study, we aimed at characterizing global changes on common cellular pathways associated with Parkinson's disease (PD) and colorectal cancer (CRC). We used gene expression data retrieved from an idiopathic PD (IPD) patient cohort and from CRC cells with unmodified versus genetically altered clocks. Our results highlight common differentially expressed genes between IPD patients and cells with disrupted clocks, suggesting a role for the circadian clock in the regulation of pathways altered in both pathologies. Interestingly, several of these genes are related to cancer hallmarks and may have an impact on the overall survival of colon cancer patients, as suggested by our analysis.

Abstract: Increasing evidence suggests a role for circadian dysregulation in prompting disease-related phenotypes in mammals. Cancer and neurodegenerative disorders are two aging related diseases reported to be associated with circadian disruption. In this study, we investigated a possible effect of circadian disruption in Parkinson's disease (PD) and colorectal cancer (CRC). We used high-throughput data sets retrieved from whole blood of idiopathic PD (IPD) patients and time course data sets derived from an in vitro model of CRC including the wildtype and three core-clock knockout (KO) cell lines. Several gene expression alterations in IPD patients resembled the expression profiles in the core-clock KO cells. These include expression changes in *DBP*, *GBA*, *TEF*, *SNCA*, *SERPINA1* and *TGFB1*. Notably, our results pointed to alterations in the core-clock network in IPD patients when compared to healthy controls and revealed variations in the expression profile of PD-associated genes (e.g., *HRAS* and *GBA*) upon disruption of the core-clock genes. Our study characterizes changes at the transcriptomic level following circadian clock disruption on common cellular pathways associated with cancer and neurodegeneration (e.g., immune system, energy metabolism and RNA processing), and it points to a significant influence on the overall survival of colon cancer patients for several genes resulting from our analysis (e.g., *TUBB6*, *PAK6*, *SLC11A1*).

Keywords: circadian rhythms; colorectal cancer; Parkinson's disease; high-throughput data; gene expression; bioinformatics; core-clock knockouts; survival analysis

1. Introduction

Several pathologies develop with aging. Among those, cancer and neurodegenerative disorders affect (in total) about 41.3 million people worldwide and are a major health problem [1,2]. These two disease groups seem to relate to opposite cellular fate phenotypes, which contribute to the pathological development. While cancer is linked to increased cellular proliferation and resistance to apoptosis, neurodegeneration involves the progressive death of neuronal cells [3]. In particular, tumorigenesis is characterized by the disruption of key molecular events, defined as the hallmarks of cancer [4]. These include aberrant cellular proliferation, resistance to cell death, induction of angiogenesis and metastasis, altered cellular energetics and evading immune system response. Neurodegeneration, on the other hand, is characterized by the excessive loss and malfunctioning of neurons, induced cell death and proteopathies such as accumulation of misfolded proteins [5]. The placement of neurodegeneration and cancer at seemingly opposite ends of specific disease mechanisms is further evidenced by the inverse correlation between cancer incidence and neurodegenerative disorders like PD that has been reported in a number of epidemiological studies [6–8]. However, more recently, it has been reported that PD patients are at greater risk of certain types of neoplasms, particularly of melanoma and brain cancer [9], suggesting putatively shared pathological mechanisms. In both diseases, the pathological development process requires a multistep accumulation of events that lead to irreversible cellular changes, as it is expressed in a linear relationship between age and increased incidence rate [10]. The hallmarks of physiologic aging and neurodegeneration include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [11,12]. Many of these processes are also relevant to tumorigenesis [3,6,13,14]. Genetic forms of PD are particularly interesting in this overlap. Parkin, an E3-ubiquitin ligase that regulates protein metabolism, mitochondrial function, mitophagy and redox homeostasis, responsible for one of the most common familial forms of PD and also implicated in IPD (PD of unknown genetic origin), is mutated in many somatic cancers, where it seems to act as a tumour suppressor gene [15]. Other genes implicated in genetic forms of Parkinsonism, like *PINK1* (PTEN-induced kinase 1), *LRRK2* (Leucine-rich repeat kinase 2), *PARK7* (Parkinsonism associated deglycase, also known as DJ-1) have also been shown to be relevant in the development of neoplasms, where they interfere in the regulation of processes such as cell cycle, apoptosis, mitochondrial homeostasis and oxidative stress. *SNCA* which encodes for α-synuclein in humans is one of the main proteins linked to Lewy bodies, which constitute the neuropathological hallmark of PD. Interestingly, α-synuclein is overexpressed in melanoma and other cancers. On the other hand, genes primarily implicated in cancer-related pathways, such as *TP53*, *MTOR*, *TSC1/2*, *PIN1*, *MC1R* and the *PTEN* pathways, have also been shown to be implicated in PD pathogenesis [16]. Thus, increasing evidence points to the disruption of common cellular events in both cancer and PD [2–5], but the mechanisms underlying these associations require further studies. These mechanisms include the regulation of cell cycle, DNA repair, immune response and redox homeostasis [3,6,13,14]. Recent work from our group using time series data for an in vitro model of colorectal cancer (CRC) progression pointed to a link between cancer and neurodegenerative diseases, involving the circadian system [17], and suggested that altered oscillatory patterns of cancer-associated genes may also be involved in neurodegeneration in Huntington's disease, but also revealed differential enrichment of genes involved in Alzheimer's disease and PD.

PD is the second most common neurodegenerative disorder, and it is expected to affect over 12 million people in the next 20 years [18]. Clinical features of PD include both motor (e.g., muscular rigidity) and nonmotor (e.g., fatigue and musculoskeletal pain) symptoms. There is currently no cure nor preventive treatment for PD. Despite the existence of multiple treatment strategies, motor disability progresses over time and nonmotor symptoms are frequently difficult to control [19]. A PD patient is also at increased risk of developing other clinical complications, which include sleep disorders [20], neuropsychiatric disorders [21], skin and brain cancer [9]. The underlying mechanisms leading to these associations are far from being understood.

Work from our group and others pointed to the involvement of the circadian system in the regulation of neurodegenerative pathways in the context of cancer cellular models [17,22], reviewed in [23]. The circadian clock is an internal timekeeping molecular machinery that generates oscillations in the expression of genes and proteins with a period of approximately 24 h [24,25]. Its proper functioning ensures appropriate timing of physiological processes such as regulation of hormonal activity, core body temperature, sleep/wake cycles and metabolism. At the molecular level, the circadian clock consists of transcriptional and translation feedback loops (TTFLs) involving several families of genes and proteins (CLOCK, BMAL, RORs, PERs, CRYs, REV-ERBs) [25,26]. These elements form the core-clock and drive 24-h rhythmic oscillations in the expression of various target genes known as clock-controlled genes (CCGs). CCGs are involved in the regulation of numerous cellular processes including DNA damage response [27], cell cycle [28–30], immune system [31], cell fate decisions [32] and metabolism [33]. The disruption of proper functioning of the circadian clock was found to be associated with several diseases including cancer [34,35], cardiovascular diseases [36], sleep disorders [37], and neurodegeneration [38].

Circadian misalignment in PD has been reported, both clinically and through animal models [39–42]. It can contribute to the appearance of disabling symptoms, both motor and nonmotor, but it can also play a crucial role in the physio-pathological mechanisms that lead to progressive neurodegeneration, providing novel symptomatic or even preventive therapeutic approaches. A recent study suggested that circadian disturbances, measured through actigraphy, antedate and predispose the development of PD during longitudinal follow-up in older men who had not been previously diagnosed with PD [43]. Whether these early changes constitute true risk factors or the manifestations of PD in its preclinical form [44] remains to be elucidated. Evidence of circadian dysfunction in PD was found also at the molecular level. Single nucleotide polymorphisms of several clock-related genes have been shown to be associated with particular PD phenotypes, like tremor-dominant (ARNTL rs900147 variant), postural instability/gait difficulty (PER1 rs2253820 variant) [45], presence of motor fluctuations, sleep disorders (CLOCK 3111T/C variant) [46] and depression (Tef rs738499 variant) [47].

The circadian expression profile of clock genes has been seldomly evaluated in PD. In a cohort of 17 PD patients *BMAL1*, *BMAL2*, *DEC1*, *CLOCK* and *PER1* expression were evaluated during the night, at four time points, from 21 h to 9 h, and compared between PD patients and a healthy control group (sex and age matched) [48,49]. The data showed a significant decrease in *BMAL1* and *BMAL2* expression at 21 h and 24 h (midnight) in PD patients compared to a control group of healthy subjects for the same time point. However, all other clock genes evaluated (*CLOCK*, *DEC1* and *PER1*) showed no significant differences. Breen and colleagues [50] quantified the expression of *BMAL1*, *PER2* and *REV-ERBα* in 30 newly diagnosed PD patients, during the day with three-hour intervals. The authors reported a lack of diurnal variation in *BMAL1* expression in PD. Furthermore, Pacelli and colleagues quantified the expression of various clock genes (*CLOCK*, *CRY1*, *CRY2*, *NR1D1*, *PER1*, *PER2*, *PER3*, *BMAL1*) in cultured fibroblasts from two PD patients with *PRKN* (Parkin) mutations and found an absence of circadian rhythmicity (period close to 24 h) particularly in *CLOCK*, *PER1* and *PER2* in fibroblasts from PD patients [22].

The mechanisms that underlie the relations between PD and the circadian clock are unknown, but they may include reciprocal regulations between clock genes and dopamine, interactions with the orexinergic system, oxidative stress and mitochondrial function and inflammation [51,52]. Visual dysfunction, extensively demonstrated in PD, may also play a role in circadian changes, given the fundamental role of light as an entrainment stimulus [53].

To further investigate the underlying role of the circadian clock in PD and cancer, we analysed microarray data sets obtained from a cohort of 205 IPD patients and 233 healthy controls [54]. We used colorectal cancer cell lines of different origins with distinct clock phenotypes to analyse the effects of clock disruption on cancer pathways, potentially related to PD. For this, we chose an in vitro CRC model with cells depicting a robust circadian clock (HCT116 and SW480 cell lines) and cells with a dysregulated clock (SW620, the metastatic counterpart of SW480 from the same patient, and HCT116 clock-KO cells). This allowed us also to investigate whether cancer cells of different progression stages correlate with pathways associated with clock disruption and PD.

Furthermore, and to attain a more mechanistic insight in the possible impact of circadian clock disruption in disease-associated pathways, including pathways related to cancer and PD pathogenesis, we complemented our analysis with newly generated time course RNA sequencing (RNA-seq) data sets for CRC cells including the wild type cells (HCT116^{WT}) and three core-clock knockout (KO) cell lines (*ARNTL*^{KO}, *PER2*^{KO} and *NR1D1*^{KO}). Our results show that core-clock disruptions affect average and rhythmic expression of genes involved in both cancer and neurodegeneration-related pathways. We found *HRAS* and *GBA* to be differentially rhythmic in *PER2*^{KO} cells as compared to the HCT116^{WT} cells, which are involved in cell proliferation and increased PD susceptibility, respectively [55]. In addition, we detected an overlap between genes differentially expressed upon core-clock KO in CRC and in IPD patients. These include *SERPINA1*, involved in protein metabolism and immune system, as well as *TGFB1*, a key component of TGF-β signalling pathway, which regulates cell proliferation, motility and differentiation. Furthermore, our analysis using a colon adenocarcinoma (COAD) data set retrieved from the TCGA (the cancer genome atlas) data base to assess the impact of these genes on cancer patient survival revealed four of these genes, namely *TUBB6*, *PAK6*, *SULT1A1*, *SLC11A1*, which are involved in cancer hallmarks [56], to have a significant impact on overall survival. Thus, our data reinforce the hypothesis of a common regulation of specific pathways and highlight a role for the circadian system and its disruption in cancer and PD.

New insights regarding the circadian rhythms of PD patients may thus contribute to a better understanding of the molecular basis of PD. Characterizing circadian oscillations in PD and cancer patients may provide a potential benefit for the diagnosis and monitoring of these two aging-related pathologies and can be further translated for treatment optimization.

2. Materials and Methods

2.1. Cell Culture

Human colorectal carcinoma cell line HCT116 (ATCC® CCL-247™) and their derived knockout mutants lacking *ARNTL* (also known as *BMAL1*), *PER2* or *NR1D1* (also known as *REV-ERB α*), respectively (see Section 2.2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Fetal Bovine Serum) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% Penicillin−Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. A LUNA™ Automated Cell Counter (Logos Biosystems, Anyang, South Korea) was used for cell counting and morphology analysis. All cell lines were tested for mycoplasma contamination (Mycoplasmacheck, Eurofins Genomics, Ebersberg, Germany).

2.2. CRISPR-Cas9 Knockout Generation

HCT116 core-clock knock out mutants were generated using the CRISPR-Cas9 methodology. In short, WT cells were transfected with CRISPR-Cas9 plasmids containing GFP marker and guide RNAs targeting multiple exons of *ARNTL*, *PER2* or *NR1D1* genes. Cell transfection was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA) according to manufacturer's instructions. Forty-eight hours after transfection, CRISPR/Cas9 GFP-positive cells were single-cell sorted into 96-well plates using an S3e cell sorter (Bio-Rad laboratories, Hercules, CA, USA). Cells were expanded and successful knockout clones were verified. For each knockout condition, several single clones were investigated on the DNA and RNA levels to characterize and confirm the knockout. Additionally, Sanger sequencing flanking the guide-RNA binding site was performed using the selected knockout clones in order to determine the genomic modification upon CRISPR-Cas9 editing. The KOs were further validated on the generated RNA-seq data using differential expression analysis on the ECCN (extended core-clock network) genes (Figure S1).

2.3. Sample Preparation for 45-h Time Course RNA-seq

HCT116 cells were seeded in triplicates (12-well plates, 2×10^5 cells/well). Sampling was carried out from 9 to 54 h after synchronization (via medium change) in 3-h intervals. Synchronizing time cues (e.g., light) that enable the resetting and coordination of circadian rhythms are termed *Zeitgeber* and the time after entrainment to a given *Zeitgeber* is referred as ZT (*Zeitgeber* Time) [57,58]. In our study, synchronization of CRC cells was performed via medium exchange (as *Zeitgeber*), which induces the activity of elements of the clock machinery and subsequently the clock-controlled genes. Of note, the time post-synchronization in cultured cancer cell lines can be compared, to some extent, to time after light exposure (related to time of the day) in humans, as light serves as the main *Zeitgeber* in mammals. To synchronize the circadian clock in cell culture environment, different synchronization agents can be used (e.g., dexamethasone, forskolin and serum shock) [28,59–61]. We have previously shown that medium exchange is as effective as other mentioned agents in synchronizing the circadian clock in different colorectal cancer cell lines as measured by the *BMAL1*-promoter activity over time [62]. Based on these results, we used a simple medium exchange to synchronize the cells. Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure RNA concentration. RNA was stored at −80 °C until further usage mRNA libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to guidelines and sequenced at the European Molecular Biology Laboratory (EMBL) GeneCore Facility (EMBL, Heidelberg, Germany) on an Illumina NextSeq 500 platform (average depth: 100 M; 75-bp paired-end reads).

2.4. RNA-seq and Microarray Data Pre-Processing

Quality control of raw reads was carried out for HCT116 cells (Accession number: E-MTAB-9701) and SW480 (ATCC® CCL-228™), SW620 (ATCC® CCL-227™) (Accession number: E-MTAB-7779 [63]) cells RNA-seq data (75 bp paired-end) using FastQC (v0.11.7) [64]. Trimmotatic (v0.38, [65]) with TruSeq3-PE-2 was used to remove adapter sequences. STAR (v2.6.0a, [66]) algorithm was used to align the reads to the human genome (Homo_sapiens.GRCh38, Ensembl release 92). Translation to transcript coordinates was carried out with quantMode TranscriptomeSAM. and further quantification was performed using Salmon (v0.10.2, [67]) with default parameters and using the seqBias option. Transcripts per million (TPM) counts were scaled using the *tximport* package (v1.6.0, [68]) by first multiplying TPM with feature length and then scaling up to the library size (lengthScaledTPM), resulting in summarized gene-level (txOut = FALSE, based on Ensembl Transcript IDs) count estimates. Log2-transformation was performed using the cpm function and applying the TMM method via the R package edgeR (v3.20.9, [69]). Only genes with $\geq$0.5 CPM (average over all time points) were retained and counts were renormalized. Raw microarray dataset (GEO Accession number: GSE99039; date of data retrieval: 16.02.2021) consisting of data sets from 205 patients with IPD and 233 controls was downloaded using R package GEOquery [70]. Expression levels were calculated using the Robust Multi-Array Average (RMA) preprocessing procedure as implemented in R affy package [71]. The R package arrayQualityMetrics [72] was used for quality control and statistical testing of the arrays. After quality control (QC) to identify possible outliers, 6 arrays failed 2 or more QC tests and were removed. Following the removal of outliers and of 1 control participant with *PINK1* mutation, 204 IPD and 227 controls were retained for further analysis (Table S1). The data was then batch corrected using ComBat [73] and annotated using the hgu133plus2.db package (version 3.2.3, [74]). For genes that mapped to multiple probes, the probe with the highest mean expression was retained. A low expression filter was set based on the histogram of intensity distribution of genes. Genes that did not pass this expression cutoff (<4) in at least 204 samples (the smaller group of samples in the cohort) were removed.

2.5. Rhythmicity Analysis

Circadian and ultradian rhythms were detected with the RAIN algorithm [75]. Circadian-related parameters (phase and amplitude) were determined using the harmonic regression method as implemented in the R package HarmonicRegression (v1.91) [76], and setting the period to 24 h for circadian and 12 h or 8 h for ultradian rhythms. For the RNA-seq data sets (E-MTAB-9701 and E-MTAB-7779), unlogged (not log2-scaled) expression values were used as input. RAIN p-values were Benjamini-Hochberg (BH) adjusted for multiple testing. Statistical significance for 24 h rhythmic genes was set at $q < 0.05$ and a relative amplitude ≥ 0.1. Amplitudes reflecting the distance between maximum and minimum data values were estimated using harmonic regression based on the formula A= $\sqrt{}$ (a2 + b2) and phases reflecting the peak expression time formulated as $\tan \varphi = b/a$. The DODR (Detection of Differential Rhythmicity) [77] method was used for differential rhythmicity analysis, and pairwise comparisons between HCT116^{WT} and each KO cell line were carried out. DODR p-values were BH adjusted for multiple testing and a threshold set for differentially rhythmic genes as $q < 0.05$.

2.6. Differential Expression Analysis

For the PD microarray datasets, differential expression analysis was performed using the R limma package [78]. A nominal $p < 0.01$ and a |log2 fold change (FC)| ≥ 0.1 was set for statistical significance of up- and down-regulation in gene expression. For RNA-seq datasets, a linear model including circadian harmonics was fitted on TMM normalized and mean-variance assessed (with voom) data. Subsequently, the coefficient representing the mean gene expression was used for comparisons of each knockout to the HCT116^{WT} condition using the R limma package. Statistical significance was set to $q < 0.05$ and |log2 FC| > 0.58 (corresponding to a |FC| > 1.5) for comparisons using only RNA-seq data, whereas a cutoff of $p < 0.01$ and an absolute $\log_2$ FC ≥ 0.1 was selected for comparison using both RNA-seq and microarray data sets. For the visualization of heatmaps, gene expression values were standardized using z-score transformation formulated as follows: $\mathrm{z-score} = \frac{\mathrm{expression\ value\ (x)} - \mu\ \mathrm{(mean)}}{\sigma \mathrm{(standard\ deviation)}}$.

2.7. Curation of KEGG Pathways

KEGG pathways known to be disrupted in cancer and neurodegeneration were pre-selected for the subsequent analysis. These included the "immune system" module, all pathways from "replication and repair" under "genetic information and processing" module and pathways from "cell growth and death" categorized under the "cellular processes" module [79,80]. Because there were no explicit redox homeostasis pathways in KEGG, we included human "energy metabolism" pathways and "transport and catabolism" pathways classified under "cellular processes". In addition, we included the PD pathway (hsa05012) from the KEGG human disease modules. The KEGG PD pathway includes elements from several other KEGG pathways (calcium signalling; unfolded protein response; mitophagy; apoptosis; dopamine metabolism; oxidative phosphorylation; microtubule-based transportation and transcription). For curation of lists, we used KEGGREST (v1.30.1, [81]) as implemented in R package. Following the curation of the results, a unique list of genes was generated comprising 2507 unique genes.

2.8. Functional Enrichment

A functional enrichment analysis was performed with GO terms (biological process—BP) for significantly rhythmic genes in CRC cells and for differentially expressed genes in CRC cells and IPD datasets. The enrichment was performed using the clusterProfiler R package [82]. The corresponding results are provided in Tables S8–S10.

2.9. Clinical Characteristics of the Cohort of IPD Patients

According to the original study of Shamir and colleagues [54], all IPD patients were subjected to an extensive clinical evaluation in a tertiary referral central and met the United Kingdom Parkinson's Disease Society Brain Bank Criteria except that positive family history was not set as an exclusion criterion. Patients with single-photon emission computer tomography (SPECT) data available were excluded from the IPD cohort if the dopaminergic deficits were not evident in the dopamine transporters scan (DaTSCAN). The raw microarray data sets (GEO Accession number: GSE99039, date of data retrieval: 16.02.2021) were retrieved from the Gene Expression Omnibus (GEO) repository. Demographic information on age and sex for controls and IPD patients, as well as age at disease onset, Hoehn and Yahr stages, and MoCA scores (Montreal cognitive assessment), are indicated for the complete dataset (GSE99039) in Table 1. Hoehn and Yahr (H and Y) scale and MoCA scores are indicated in Table 1. H and Y scale reflects the overall progression of disabilities and symptoms in PD, with higher scores indicating higher disability (ranging from 1: unilateral involvement only with minimal or no functional disability; to 5: confinement to bed or wheelchair unless aided) [83]. The MoCA test is a rapid screening instrument for mild cognitive dysfunction that assesses different cognitive domains. The total possible score is 30 points, and a score of 26 or above is considered normal.

Table 1. Clinical characterization of the IPD patients. MoCA; NA = Not Applicable. The clinical characteristics listed in Table 1 correspond to the entire dataset downloaded (GEO: GSE99039). For the subsequent analysis, only 204 PD patients and 227 controls were retained (see Materials and Methods, Section 2.4 for further details).

Sample Size	205 IPD Patients	233 Controls
Sex	101 males; 90 females; 14 samples without sex information	70 males; 142 females; 21 samples without sex information
Age (all patients)	62 ± 11 (mean ± SD)	58 ± 30 (mean ± SD)
Age at disease onset (all patients)	56 ± 11 (mean ± SD)	NA
MoCA	27 ± 3	NA
Hoehn and Yahr stage		
0	7	NA
1	58	NA
2	70	NA
3	30	NA
4	8	NA
5	0	NA
Missing data	32	NA

2.10. TCGA Colon Adenocarcinoma (TCGA-COAD) Analysis

Colon adenocarcinoma RNA-Seq data sets (COAD, HiSeq 2000) and corresponding clinical information were retrieved from TCGA (available at https://portal.gdc.cancer.gov, Accessed date: 8 August 2021) using the TCGAbiolinks package [84,85]. Primary tumour (N = 285) and normal tissue samples (N = 41) were included in the analysis. Gene expression quantification files aligned to Human Genome (Homo sapiens, GRCh37, hg19) were retrieved for further processing from the legacy archive. Raw counts were normalized using TMM from edgeR package [69] and voom functionality from the limma package [78] used for assessment of the mean variance relationship of log-counts. A low gene expression filtering (logCPM > 0.5, in more than 50% of the total population) was used to define the gene expression cutoff. Following batch correction, assessed based on the plate information, differential expression analysis was performed using the limma package [78]. The p-values were BH adjusted for multiple testing and a cutoff of log FC > 1 and FDR $q < 0.01$ was used to determine the significance of differential expression (DE) by comparing the cancer tissue versus samples derived from the normal adjacent tissue. The resulting 3234 DE genes were subsequently analysed using Kaplan–Meier (KM) survival analysis to compute survival univariate curves. Out of the DE gene list, 289 were significantly associated with patient overall survival ($q < 0.05$). The clinical features regarding the last follow-up and days until death were used to assess the survival analysis for cancer tissue samples. Briefly, each gene expression value in cancer samples was divided into two groups based on the median expression level (high versus low expression) in all samples. KM survival analysis was performed between high and low expression groups using the 'survival' package (version 3.2-12). The fitted survival curves were visualized using survminer package (version 0.4.9). We additionally investigated the possible influence of clinical traits (sex, age, cancer stage) using the TCGA COAD datasets. Because stage correlated significantly with the survival data (Figure S15), we used a Cox Regression model by including stage as a stratification factor (the list of survival associated genes is provided in Table S12).

3. Results

3.1. The Disruption of Core-Clock Genes Alters the Rhythmic Expression of Genes Involved in Both Cancer- and Neurodegeneration-Related Pathways

Cancer and neurodegeneration share altered cellular processes including disruption of cell cycle, DNA repair, immune system and redox homeostasis [3,17,86]. To investigate possible circadian clock-related alterations in the expression of elements of these pathways, we gathered 438 microarray data sets retrieved from whole blood samples of IPD patients [54] (GEO: GSE99039), 64 RNA-seq data sets from CRC cell lines (newly generated data sets for HCT116^{WT} and three HCT116 derived-KO cells (ArrayExpress: E-MTAB-9701) and previously generated 22 RNA-seq time course data sets for two additional CRC cell lines originated from the primary tumour and metastasis site of the same patient (SW480, SW620; ArrayExpress: E-MTAB-7779) and known to have different clock phenotypes [63]. The PD data (GEO: GSE99039) was obtained from whole blood of 205 patients (90 females, 101 males and 14 samples without sex information) with IPD and 233 controls (142 females, 70 males and 21 samples without sex information) (Figure 1, right panel and Table 1). We complemented the IPD data sets with newly generated data sets from three core-clock KO mutants (HCT116-*ARNTL*^{KO}, HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO}) and the corresponding WT cells, as well as previously published data sets from two CRC cell lines as indicated above, to investigate possible clock-related alterations relevant in cancer development and neurodegeneration-related genes with a particular focus on PD. For all four cell lines (HCT116^{WT} and HCT116-derived KOs), we generated RNA-seq time course data (Figure 1, left panel).

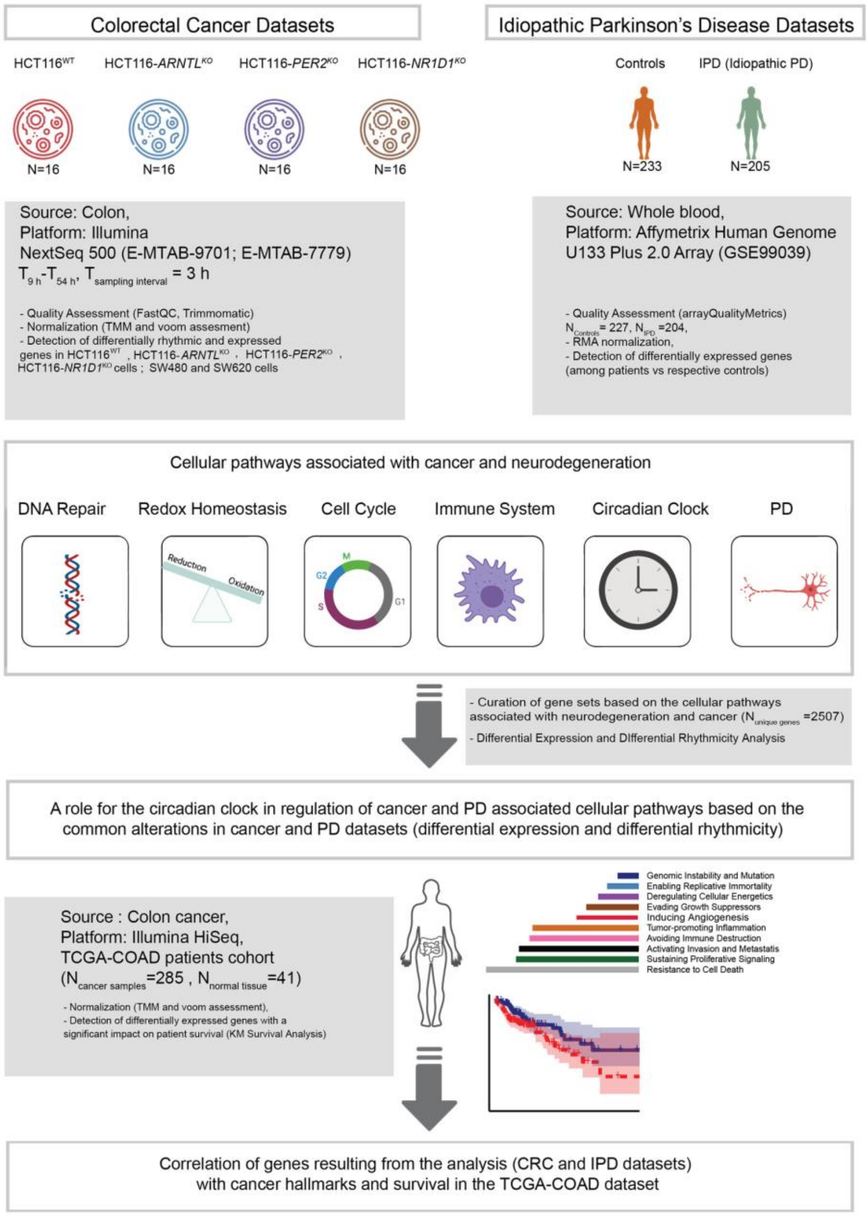


Figure 1. Workflow for the analysis of time-series RNA-seq data sets from CRC cell lines and microarray data sets from a cohort of IPD patients. KO cells were derived from HCT116^{WT} cells using CRISPR/Cas9 methodology. RNA-seq data sets (ArrayExpress: E-MTAB-9701) include the HCT116^{WT} and three core-clock knockout cells (HCT116-*ARNTL*^{KO}, HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO}) sampled as indicated. Data sets of two additional CRC cell lines (SW480, SW620; ArrayExpress: E-MTAB-7779) were also included in the rhythmicity analysis as indicated. Following data pre-processing, circadian and ultradian rhythms were detected and differential rhythmicity analysis was performed. In addition, differential gene expression analysis was carried out for all expressed genes as compared to the corresponding controls, for HCT116 cells and IPD data sets (GEO: GSE99039).

For the time-course data sets, we performed a rhythmicity analysis for genes oscillating with circadian (24 h) and ultradian periods (8 h and 12 h) and a subsequent differential rhythmicity analysis to characterize KO-related variations in the rhythmic expression phenotype (loss of oscillations or change in phases and/or amplitudes). In addition, and to allow for data comparison between the IPD (single time point data sets) and the CRC data (time-course data sets), we performed a differential expression analysis to investigate putative alterations in the mean gene expression levels in the HCT116 cells and the PD data.

We focused our analysis on molecular mechanisms, commonly disrupted in both cancer and PD-related pathways. For this, we curated a gene list based on a literature search on commonly disrupted pathways in both cancer and neurodegeneration with a focus in PD, which included several KEGG pathways, namely immune system (Table S2), DNA damage (Table S3), cell cycle (Table S4), redox homeostasis (from energy metabolism and catabolism pathways) (Table S5), a published list of core-clock genes comprising the core-clock network (CCN) and the ECCN [87,88], (Table S6), and the PD pathway (Table S7). The last one was complemented based on a literature curation of published studies describing additional genes involved in genetic forms of PD or linked to increased risk of symptoms with possible circadian influences (motor fluctuations, hallucinations and psychosis, sleep symptoms including REM behavior disorder or restless legs syndrome) (Table S7). This resulted in 2507 unique genes, named hereafter as "genes of interest".

To study the influence of clock dysregulation on the genes of interest, we performed a rhythmicity analysis in HCT116^{WT} and the three KO cell lines. As expected, for the KO cells we observed a reduction in the overall number of rhythmic genes (both circadian and ultradian). In addition, we observed a variation in the global phase distribution in all HCT116^{KO} conditions when compared to HCT116^{WT} (Figure 2A,B,E,F, density plots). HCT116^{WT} cells showed a bimodal distribution for genes expressed with 8-h and 24-h periods, whereas a multimodal distribution was observed for genes expressed with a period of 12 h, and this pattern was lost in all KOs (Figure 2A,B,E,F). For the genes of interest, we plotted the phase and amplitude of rhythmic genes in each KO cell line (Figure 2A,B,E,F, scatter plots). All three KOs led to a decrease in the number of rhythmic genes (within the set of genes of interest), as expected (HCT116^{WT}: $N_{24h\sim GenesofInterest} = 143$, $N_{12h\sim GenesofInterest} = 27$ and $N_{8h\sim GenesofInterest} = 1$; HCT116-*ARNTL*^{KO}: $N_{24h\sim GenesofInterest} = 35$, $N_{12h\sim GenesofInterest} = 16$; $N_{8h\sim GenesofInterest} = 5$; HCT116-*PER2*^{KO}: $N_{24h\sim GenesofInterest} = 52$, $N_{12h\sim GenesofInterest} = 2$, $N_{8h\sim GenesofInterest} = 4$; HCT116-*NR1D1*^{KO}: $N_{24h\sim GenesofInterest} = 76$, $N_{12h\sim GenesofInterest} = 10$ and $N_{8h\sim GenesofInterest} = 3$) (Figure 2A,B,E,F). Most of the rhythmically expressed genes of interest were circadian expressed (66%), whereas only 33% showed 12 h rhythms, in HCT116^{WT} cells. Out of the rhythmic set of genes of interest, only 1% had 8 h periods in HCT116^{WT} cells. In all KO conditions, a similar pattern was observed in the proportion of rhythmic genes of interest oscillating with different periods. The majority of genes oscillated with a 24 h period.

Moreover, the oscillatory characteristics of the genes of interest depicted in the scatter plots (amplitudes, and phases estimated by the rhythmicity analysis) resembled those observed in the density plots for the total number of oscillating genes, with a bimodal distribution of phases for 24 h rhythmic genes in HCT116-*ARNTL*^{KO} cells (Figure 2B) and a multimodal distribution of phases (0–8 h; 8–16 h; 16–24 h) in HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} cells (Figure 2E,F). This indicates that our pre-selected genes of interest show alterations in rhythmicity upon core-clock manipulation, which reflect the global expression changes in each KO cell line.

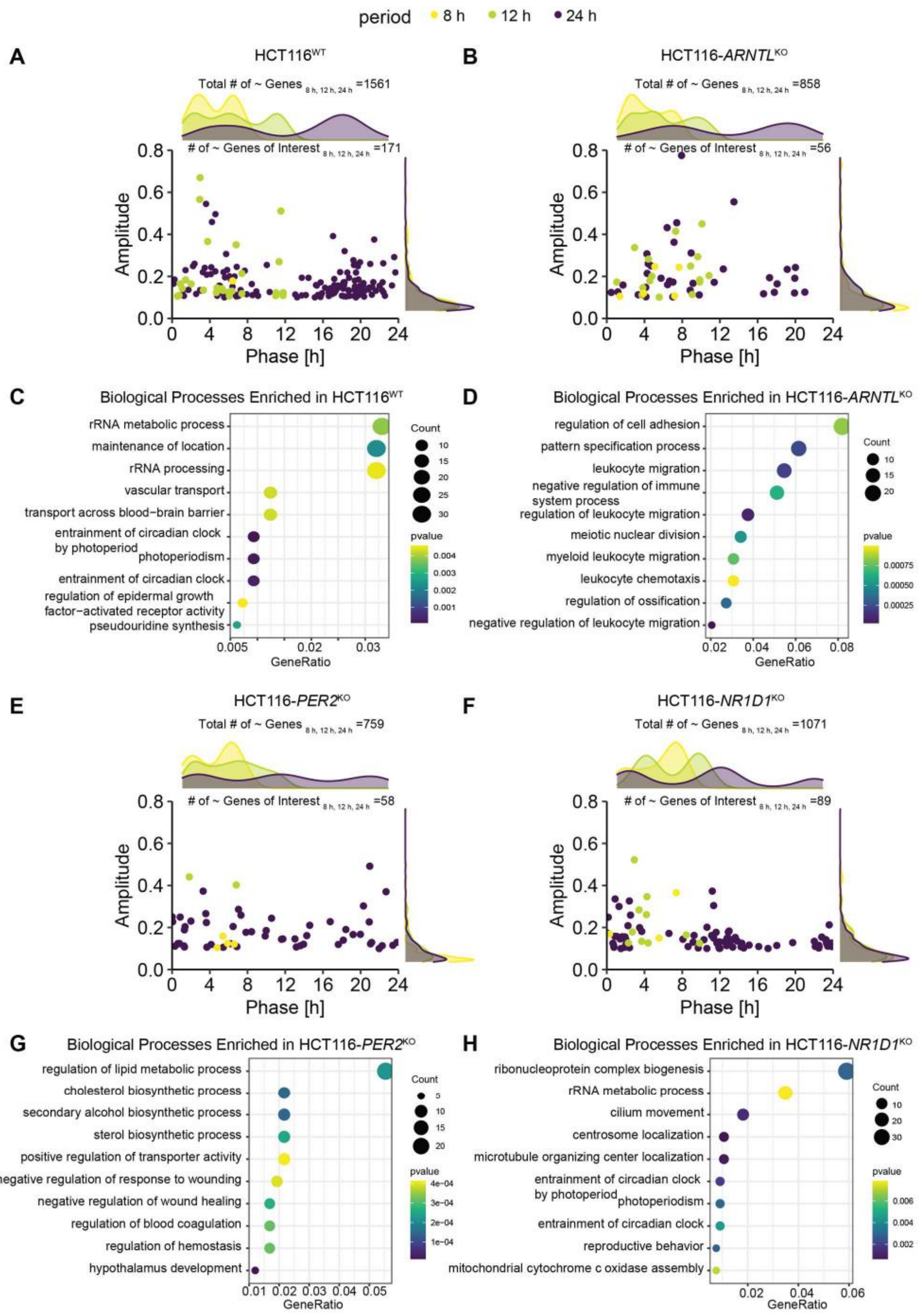


Figure 2. Core-clock KO affects the rhythmic characteristics of expressed genes in HCT116 cells and the associated biological

processes. (**A**) Acrophase and amplitude distribution of significantly oscillating genes (based on RAIN $q < 0.05$ and relative amplitude ≥ 0.1) in HCT116^{WT} cells ($N_{total\sim 8\,h,12\,h,24\,h} = 1561$, $N_{total24\,h\sim genes} = 1241$, $N_{total12\,h\sim genes} = 273$, $N_{total8\,h\sim genes} = 46$; $N_{totalGenesofInterest\sim 8\,h,12\,h,24\,h} = 171$, $N_{24\,h\sim GenesofInterest} = 143$, $N_{12\,h\sim GenesofInterest} = 27$ and $N_{8\,h\sim GenesofInterest} = 1$), (**B**) Acrophase and amplitude distribution of significantly oscillating genes in HCT116-*ARNTL*^{KO} ($N_{total\sim 8\,h,12\,h,24\,h} = 858$, $N_{total24\,h\sim genes} = 459$, $N_{total12\,h\sim genes} = 321$, $N_{total8\,h\sim genes} = 78$; $N_{totalGenesofInterest\sim 8\,h,12\,h,24\,h} = 56$, $N_{24\,h\sim GenesofInterest} = 35$, $N_{12\,h\sim GenesofInterest} = 16$; $N_{8\,h\sim GenesofInterest} = 5$), (**C**) Top 10 GO (Biological Processes) enrichment results of 24 h rhythmic genes in HCT116^{WT} cells (**D**) Top 10 GO (Biological Processes) enrichment results for 24-h rhythmic genes in HCT116-*ARNTL*^{KO} (**E**) Acrophase and amplitude distribution of significantly oscillating genes in HCT116-*PER2*^{KO} ($N_{total\sim 8\,h,12\,h,24\,h} = 759$, $N_{total24\,h\sim genes} = 539$, $N_{total12\,h\sim genes} = 179$, $N_{total8\,h\sim genes} = 41$; $N_{totalGenesofInterest\sim 8\,h,12\,h,24\,h} = 58$, $N_{24\,h\sim GenesofInterest} = 52$, $N_{12\,h\sim GenesofInterest} = 2$; $N_{8\,h\sim GenesofInterest} = 4$) (**F**) Acrophase and amplitude distribution of significantly oscillating genes in HCT116-*NR1D1*^{KO} ($N_{total\sim 8\,h,12\,h,24\,h} = 1071$, $N_{total24\,h\sim genes} = 884$, $N_{total12\,h\sim genes} = 152$, $N_{total8\,h\sim genes} = 35$; $N_{totalGenesofInterest\sim 8\,h,12\,h,24\,h} = 89$, $N_{24\,h\sim GenesofInterest} = 76$, $N_{12\,h\sim GenesofInterest} = 10$ and $N_{8\,h\sim GenesofInterest} = 3$) (**G**) Top 10 GO (Biological Processes) enrichment results for 24-h oscillating genes in HCT116-*PER2*^{KO} (**H**) Top 10 GO (Biological Processes) enrichment results for 24-h oscillating genes in HCT116-*NR1D1*^{KO}. Density plots on top panels indicate total number of oscillating genes. Number of oscillating genes of interest (for 8 h, 12 h, and 24 h periods) and total number of oscillating genes (for 8 h, 12 h, and 24 h periods) indicated above each panel. Pre-selected genes of interest are visualized in the scatter plots. The genes oscillating with different periods are colour coded (24 h~, purple; 12 h~, green; 8 h~, yellow). "~" stands for significantly oscillating genes.

To identify highly represented biological processes for rhythmic genes in HCT116^{WT} and KO conditions, we performed an enrichment analysis using the 24-h (Figure 2C,D,G,H) and 12-h rhythmic genes (Figure S2). The top 10 GO biological process in HCT116^{WT} and in HCT116-*NR1D1*^{KO} showed an enrichment of genes involved in entrainment of circadian rhythms and RNA processing (Figure 2C,H). In HCT116-*ARNTL*^{KO} (Figure 2D), the majority of top enriched biological processes were related to immune system and cell motility, whereas in HCT116-*PER2*^{KO} (Figure 2G) metabolic processes and angiogenesis pathways were among the top listed enriched biological pathways. For 12-h rhythmic genes, our analysis showed an enrichment of metabolic processes in HCT116^{WT} (Figure S2A) and HCT116-*NR1D1*^{KO} (Figure S2D), which are processes known to be rhythmic with shorter periods [33,61], whereas developmental processes were enriched in HCT116-*ARNTL*^{KO} (Figure S2B) and HCT116-*PER2*^{KO} (Figure S2C).

We further evaluated the influence of clock disruption on the overall rhythmicity profiles of the genes of interest for each KO versus the WT cells (Figure 3A–C). For this, we carried out a differential rhythmicity analysis for all genes oscillating in at least one of the conditions (WT or KO) with 24- and 12-h periodicity. No differentially rhythmic genes were found with eight-hour periods. Among the circadian genes, 13 differentially rhythmic genes were identified in all KO conditions as compared to the WT. These included the core-clock gene *CRY1*, genes crucial for transcriptional regulation, DNA repair and cell growth including *HLF* (transcription factor, related to the PAR BZIP family and associated with leukaemia), *POLD1* (DNA polymerase associated with colorectal cancer), *MCM2* (mini-chromosome maintenance protein regulating DNA replication), *TP73* (a member of TP53 family and a candidate gene for neuroblastoma) in which one of the isoforms, ΔNp73, is known to function as a pro-survival factor in neurons whose haploinsufficiency might be a risk factor for neurodegeneration [89–91], histone protein variants *H2AW* and *H2AX, TELO2* (S-phase checkpoint regulator), and important regulators of redox homeostasis like *NOS3,* which acts as a mediator in several biological processes, including neurotransmission and antitumoral activities, *NDUFA11* involved in the mitochondrial electron transport chain, and microtubules structural components like *TUBB, TUBB2A, TUBB4B*, which regulate intracellular transport and neuronal communication (Figure 3A–C). These genes depicted significant oscillations in expression in HCT116^{WT} cells, whereas their rhythmicity was disrupted in the KO cells. The KO of *ARNTL* led to the strongest effect with loss of rhythmicity for all genes listed above (Figure 3A). In addition, each KO showed a unique set of differentially rhythmic genes of interest. Among the differentially rhythmic genes unique to HCT116-*ARNTL*^{KO} were the transcriptional regulator genes *DBP* and *TEF*, which showed a disruption of rhythmicity, mitochondrial- and lysosomal-energy-metabolism-related genes *NDUFS6*, *NAGLU*, *UQCR10,* genes related to cellular growth and DNA repair mechanisms *RAD51B, RNASEH2C,* inhibitor of TNF-induced apoptosis gene *TNFIAP3*, and *PIN1*, which regulates genotoxic and other stress responses, as well as cellular growth (Figure 3A). The majority of differentially rhythmic genes specific to HCT116-*PER2*^{KO} are involved in the regulation of DNA damage response, cell cycle and apoptosis (*ABRAXAS1, AIFM2, BUB3, HIPK3, HRAS, HRK, MAPK12, SIVA1, TAB1* and *TFE3*) (Figure 3B). Moreover, two well-known clock genes, *NR1D1* and *BHLHE41* (also known as *DEC2*, a clock gene that interacts with ARNTL or competes for E-box binding sites in the promoter region of *PER1*) were also differentially rhythmic uniquely in HCT116-*PER2*^{KO} (Figure 3C). In addition, genes involved in the regulation of energy metabolism such as *GBA* (a lysosomal membrane protein involved in glycolipid metabolism), which is found to be frequently mutated in PD patients and linked to increased disease susceptibility, *ACSL1, CAMK2D, MCOLN1* and *MVK*, genes involved in immune system activation and immune cell differentiation such as *CD55* and *IGBP1*, the transcription regulator *POLR2E*, which encodes for RNA polymerase and *HSPA2*, a regulator of protein synthesis (protein folding, transport and proteolysis of misfolded proteins), were also identified to be differentially rhythmic only in HCT116-*PER2*^{KO}. Most of the differentially rhythmic genes unique to HCT116-*NR1D1*^{KO}, were involved in energy metabolism and regulation of ubiquitination (*ACP2, ACSL5, AGAP3, ATP5F1D, HIF1A, NDUFC2, NDUFS7, PEX16, PSMD9, UQCRQ*). In addition, several genes responsible for the regulation of genomic stability (apoptosis and DNA repair) and regulators of cellular proliferation (*FOS, GADD45 MCL2, MLST8, PPP2CA, RAC3, RRBPJ, SLK, SLX1A*) were differentially expressed (Figure 3C). Strikingly, *DRD1*, which encodes for the most abundant dopamine receptor in the central nervous system (CNS), was also differentially rhythmic in this KO, showing a phase shift between HCT116^{WT} and HCT116-*NR1D1*^{KO} (Figure 3C). Additionally, genes involved in regulation of cellular senescence (*CYBA*), cellular signalling (*ACTB, AFDN*), cellular structural organization (*ANTXR2, RAC3* and *TRIP6*), transcriptional regulator of polymerase II (*POLR2L*), immune system activity (*IL1RAP, MAVS*), *F12* (coagulation Factor XII), which functions as activator of the blood clotting cascade, as well as *TUBB2B* (major component of microtubules) were found to be uniquely differentially rhythmic in HCT116-*NR1D1*^{KO} (Figure 3C). As a comparison, we additionally analysed the expression profiles of the same genes in the CRC cell lines SW480 and SW620 and also observed variations in their circadian expression profiles (Figure S3). Taken together, these results suggest that the manipulation of core-clock genes results in alterations of rhythmicity patterns of genes involved in cancer and neurodegeneration.

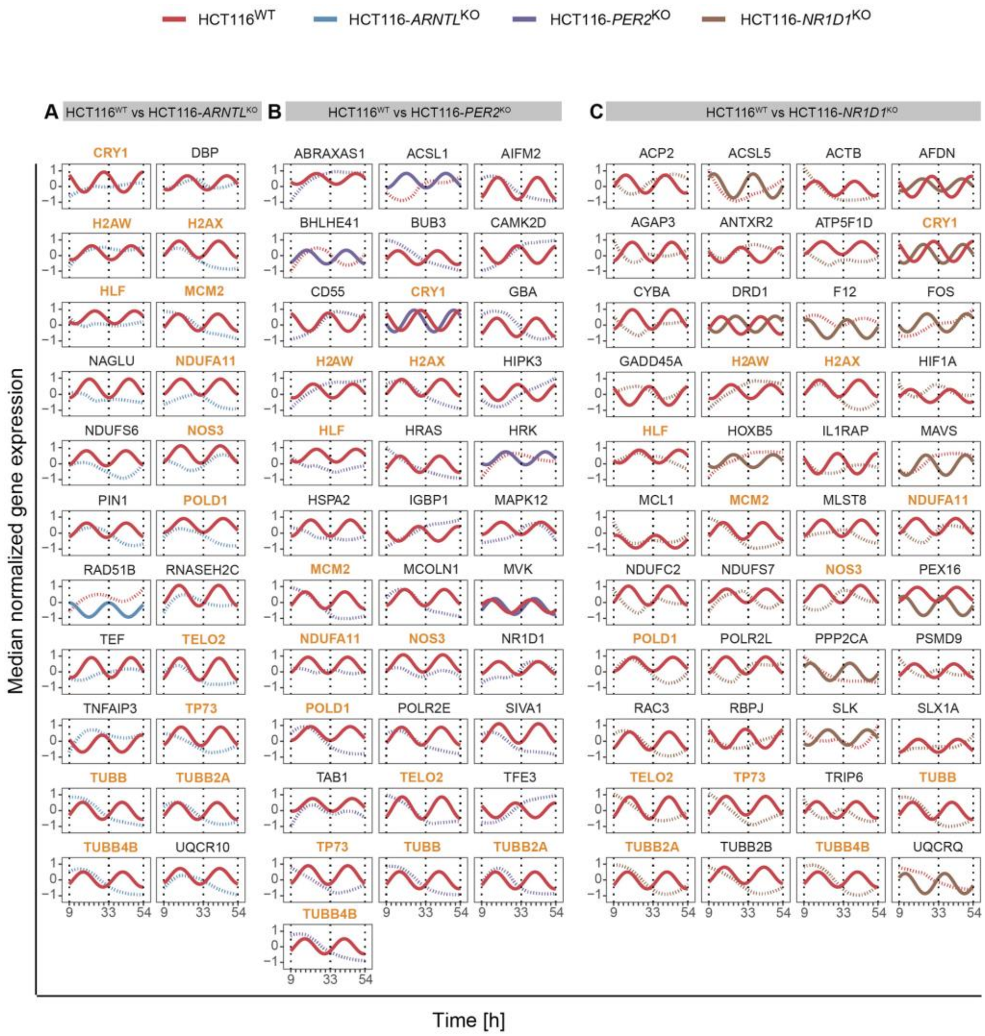


Figure 3. Circadian expression profiles of genes of interest detected as differentially rhythmic in all HCT116 KOs. (**A**) in HCT116^{WT} and HCT116-*ARNTL*^{KO} (**B**) in HCT116^{WT} and HCT116-*PER2*^{KO} (**C**) in HCT116^{WT} and HCT116-*NR1D1*^{KO}. Significantly rhythmic genes ($q < 0.05$; relative amplitude ≥ 0.1) are depicted using a harmonic regression curve (full lines); not significantly rhythmic genes are depicted with LOESS (Locally Estimated Scatterplot Smoothing) (dashed lines) (see Figure S3 for similar analysis in SW480 and SW620 cells). Common differentially rhythmic genes across all KO cells, as compared to the WT, are marked in orange.

3.2. *The Impact of Core-Clock Knockouts in CRC Cells Shows an Overlap of Differentially Expressed Genes with the Idiopathic Parkinson's Disease Cohort*

To further explore the overlap between gene expression alterations in blood-derived IPD datasets and our CRC model, we performed a differential expression analysis comparing mean gene expression levels of HCT116^{KO} to WT cells and IPD patients to a control group of healthy subjects. For IPD data sets, we divided the cohort into females' and males' subgroups to also detect possible sex-specific effects. We then performed an enrichment analysis to identify biological processes associated to the differentially expressed genes (Figures S4 and S5). Our results showed that both in the HCT116^{KO} cells, as well as in the cohort of IPD patients, the majority of top represented biological processes were related to immune system functioning (Figures S4 and S5). In addition, the observed expression changes for the genes of interest, which were commonly differentially expressed in all core-clock KOs, suggested the existence of a KO specific effect in the regulation of these genes (Figure S6). Following our analysis, we identified commonly differentially expressed genes of interest in each core-clock KO cell line and in the IPD cohort versus the respective controls (Figure 4A–C). HCT116-*PER2*^{KO} shared the highest number of genes with the same variations in a synergistic way (upregulation/downregulation) as in the IPD cohort followed by HCT116-*NR1D1*^{KO} and HCT116-*ARNTL*^{KO}. On the contrary, HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO} showed the same and also the highest number of antagonistic changes (inverse gene expression alteration compared to the IPD data sets) followed by HCT116-*ARNTL*^{KO}.

Next, we extracted commonly differentially expressed genes between the IPD and the KO cells. Out of these genes, *PXN*, a mediator of extracellular matrix organization and focal adhesion, and one of our genes of interest, was differentially expressed in both HCT116-*NR1D1*^{KO} and HCT116-*ARNTL*^{KO} cells and in the IPD cohort (Figure 4A–C). *PXN* is known to promote tumour progression in cervical cancer [92]. Additionally, *PSMB3, SIP3* and *TYK2* (unique to HCT116-*PER*^{KO}*)* and *SIPA1, ATP6V0D1* (differentially expressed only in HCT116-*NR1D1*^{KO}) were also found within the top differentially expressed genes of interest in the IPD cohort. Among the differentially expressed genes of interest, nine genes showed a change in expression levels in all KOs and IPD patients. These included *CYBA* and *LDLRAP1s*, involved in oxidase activity and endocytosis, *VASP* involved in axonal guidance and structure, *IGF2R, SERPINA1, HDAC2, TGFB1* involved in immunity and inflammation and *CDKN1C* and *SP1* involved in cell cycle and growth (Figure 4A–C). *LSP1* (intracellular F-actin binding protein regulating adhesion, motility and migration), *ATP6V0D1* (a component of vacuolar ATPase mediating acidification of intracellular organelles), *FANCF* (Fanconi anaemia complementation group F) and *VPS37B* (vacuolar protein sorting involved in calcium-dependent protein binding) were differentially expressed in the IPD cohort and HCT116-*NR1D1*^{KO} cells (Figure 4C). *CHMP7* (charged multivesicular body protein seven involved in cellular senescence), *PPA1* (member of the inorganic pyrophosphatase (PPase) family involved in phosphate metabolism of cells), *ATG16L2* (autophagy related protein), *BNIP3* (mitochondrial protein that contains a BH3 domain and acts as a pro-apoptotic factor) and *SIPA* (mitogen induced GTPase activating protein (GAP) involved in RAS pathway and innate immune system) were differential expressed in HCT116-*PER2*^{KO} cells and the IPD cohort (Figure 4B), whereas no differentially expressed genes were found common to the IPD cohort and solely unique to the HCT116-*ARNTL*^{KO} cells.

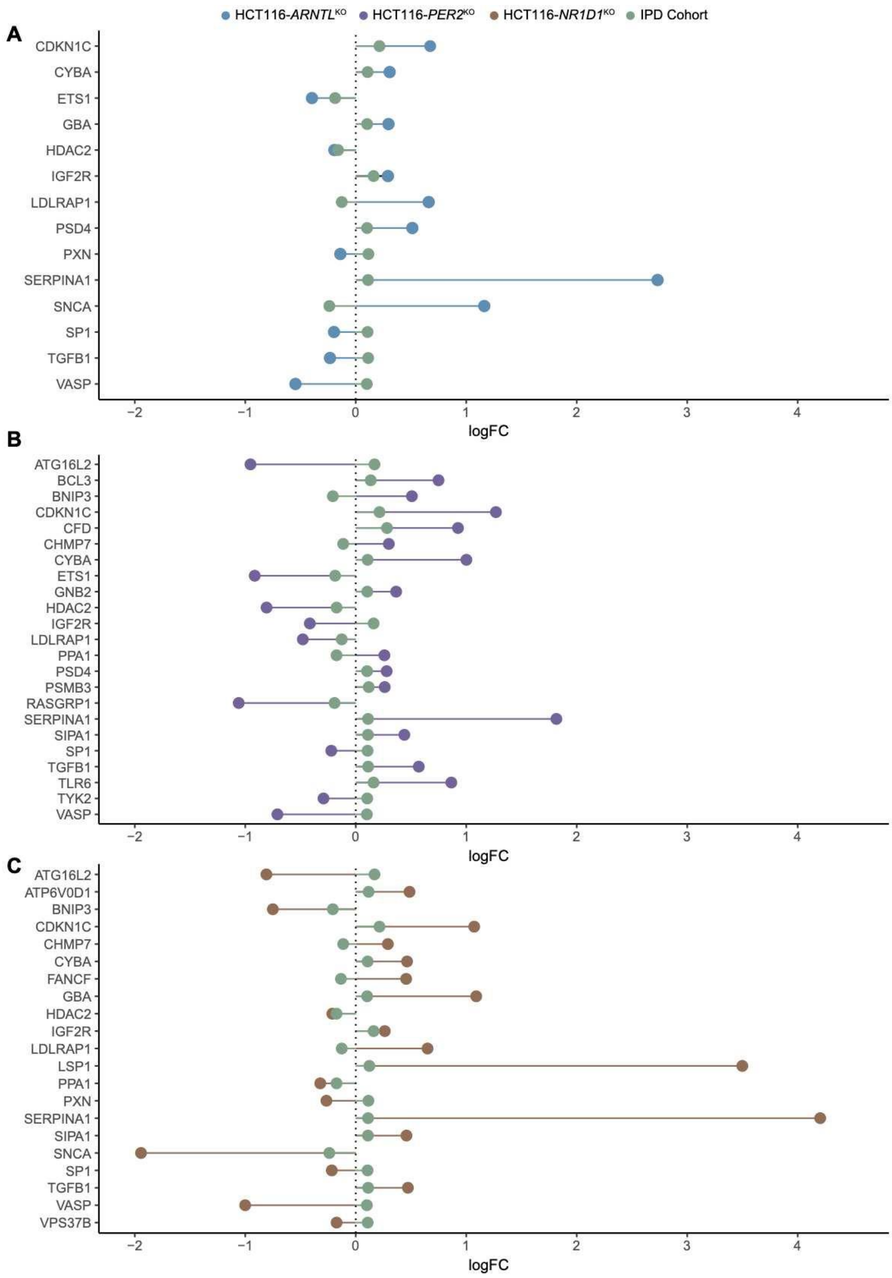
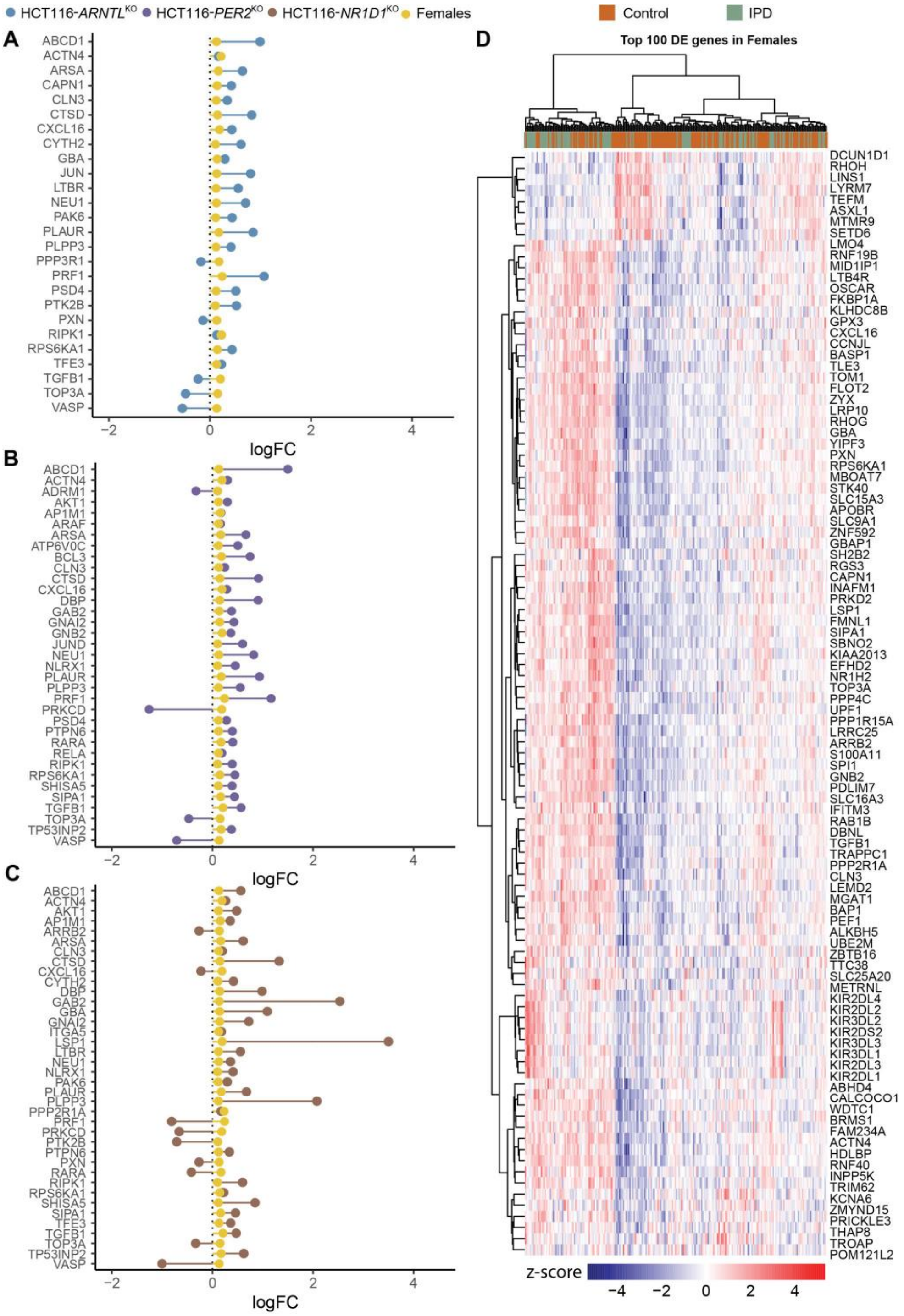


Figure 4. Common differentially expressed genes of interest in HCT116 KO cells and IPD cohort. Lollipop plots show the direction of changes (up/downregulation) in mean gene expression levels ($\log_2$ FC in comparison to respective controls) for the genes of interest shared between (**A**) HCT116-*ARNTL*^{KO} versus HCT116^{WT} and IPD patients versus controls (**B**) HCT116-*PER2*^{KO} versus HCT116^{WT} and IPD patients versus controls (**C**) HCT116-*NR1D1*^{KO} versus HCT116^{WT} and IPD patients versus controls.

We next analysed the commonly differentially expressed genes in the KO cells and the IPD cohort following sex separation ($N_{IPD\ Females}$ = 90, $N_{IPD\ Males}$ = 100 and $N_{Control\ Females}$ = 138, $N_{Control\ Males}$ = 69). The subsequent differential expression analysis resulted in less differentially expressed genes shared between KO conditions and IPD cohort for females (Figure 5A–C) as compared to males (Figures S7 and S8). *CAPN1* and *JUN*, involved in apoptosis, and *PPP3R1,* involved in the immune system, were found to be differentially expressed specifically in HCT116-*ARNTL*^{KO} and in the female patients (Figure 5A). *GNB2* (guanine nucleotide-binding protein involved in ERK signalling)*, JUND* (member of the JUN family regulating p53-dependent senescence and apoptosis)*, RELA* (*p65*), a proto-oncogene and a subunit of the classical NF-κB cascade that forms a heterodimeric complex with the p50 (NF-κB1) subunit, *ARAF* (proto-oncogene and a member of the RAF subfamily involved in cell growth and development)*, ADRM1* (adhesion regulating molecule 1 protein family regulating cell adhesion)*, BCL3* (a proto-oncogene candidate and a transcriptional co-activator that activates through its association with NF-κB) were differentially expressed in the female patients and in HCT116-*PER2*^{KO} cells (Figure 5B), whereas *LSP1* (intracellular F-actin binding protein, regulating motility, adhesion and migration)*, ARRB2* (member of arrestin/β-arrestin protein family, involved in cellular responses to stimuli)*, PPP2RIA* (subunit of protein phosphatase two, implicated in the negative control of cell growth and division) and *ITGA5* (integrin α-chain family member, which functions in cell surface adhesion and ERK signalling) were differentially expressed in both HCT116-*NR1D1*^{KO} cells and the female IPD patients (Figure 5C). In addition, the core-clock regulator *DBP* was found to be commonly differentially expressed in HCT116-*PER2*^{KO}, HCT116-*NR1D1*^{KO} and female patients (Figure 5B,C). Male IPD patients showed a higher number of differentially expressed genes of interest overlapping with the KO cells; however, more genes showed an opposite trend in their expression change compared to the KO cells, as in the female patients (Figures S7 and S8). We next analysed the top 100 differentially expressed genes in females and males. These genes did not provide a distinct genetic signature to fully distinguish controls from IPD patients (Figures 5D and S9). Nevertheless, a different genetic signature seems to be associated with either IPD female or male patients.


HCT116-*ARNTL*KO HCT116-*PER2*KO HCT116-*NR1D1*KO Females
A
ABCD1
ACTN4
ARSA
CAPN1
CLN3
CTSD
CXCL16
CYTH2
GBA
JUN
LTBR
NEU1
PAK6
PLAUR
PLPP3
PPP3R1
PRF1
PSD4
PTK2B
PXN
RIPK1
RPS6KA1
TFE3
TGFB1
TOP3A
VASP
−2 0 2 4
logFC
B
ABCD1
ACTN4
ADRM1
AKT1
AP1M1
ARAF
ARSA
ATP6V0C
BCL3
CLN3
CTSD
CXCL16
DBP
GAB2
GNAI2
GNB2
JUND
NEU1
NLRX1
PLAUR
PLPP3
PRF1
PRKCD
PSD4
PTPN6
RARA
RELA
RIPK1
RPS6KA1
SHISA5
SIPA1
TGFB1
TOP3A
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ACTN4
AKT1
AP1M1
ARRB2
ARSA
CLN3
CTSD
CXCL16
CYTH2
DBP
GAB2
GBA
GNAI2
ITGA5
LSP1
LTBR
NEU1
NLRX1
PAK6
PLAUR
PLPP3
PPP2R1A
PRF1
PRKCD
PTK2B
PTPN6
PXN
RARA
RIPK1
RPS6KA1
SHISA5
SIPA1
TFE3
TGFB1
TOP3A
TP53INP2
VASP
−2 0 2 4
logFC
D
Control IPD
Top 100 DE genes in Females
DCUN1D1
RHOH
LINS1
LYRM7
TEFM
ASXL1
MTMR9
SETD6
LMO4
RNF19B
MID1IP1
LTB4R
OSCAR
FKBP1A
KLHDC8B
GPX3
CXCL16
CCNJL
BASP1
TLE3
TOM1
FLOT2
ZYX
LRP10
RHOG
GBA
YIPF3
PXN
RPS6KA1
MBOAT7
STK40
SLC15A3
APOBR
SLC9A1
ZNF592
GBAP1
SH2B2
RGS3
CAPN1
INAFM1
PRKD2
LSP1
FMNL1
SIPA1
SBNO2
KIAA2013
EFHD2
NR1H2
TOP3A
PPP4C
UPF1
PPP1R15A
LRRC25
ARRB2
S100A11
SPI1
GNB2
PDLIM7
SLC16A3
IFITM3
RAB1B
DBNL
TGFB1
TRAPPC1
PPP2R1A
CLN3
LEMD2
MGAT1
BAP1
PEF1
ALKBH5
UBE2M
ZBTB16
TTC38
SLC25A20
METRNL
KIR2DL4
KIR2DL2
KIR3DL2
KIR2DS2
KIR3DL3
KIR3DL1
KIR2DL3
KIR2DL1
ABHD4
CALCOCO1
WDTC1
BRMS1
FAM234A
ACTN4
HDLBP
RNF40
INPP5K
TRIM62
KCNA6
ZMYND15
PRICKLE3
THAP8
TROAP
POM121L2
z-score
−4 −2 0 2 4

Figure 5. Commonly differentially expressed genes of interest in HCT116 KOs and female IPD patients. Lollipop plots show the direction of changes (up/downregulation) in mean expression levels ($\log_2$ FC in comparison to respective controls) for the genes of interest shared between (**A**) HCT116-*ARNTL*^{KO} versus HCT116^{WT} and female IPD patients versus respective controls; (**B**) HCT116-*PER2*^{KO} versus HCT116^{WT} and female IPD patients versus respective controls; (**C**) HCT116-*NR1D1*^{KO} versus HCT116^{WT} and female IPD patients versus respective controls. (**D**) Heatmap of top 100 differentially expressed genes (sorted by *p*-value) for IPD female patients. The heatmap was clustered using WardD2 linkage and Euclidean distancing. Disease status is depicted as orange for controls and green for patients. Colour code scheme for standardized gene expression values (z-scores) are indicated in the heatmap colour key (see Figures S7–S9 for similar analysis in males).

3.3. The Relative Expression among ECCN Elements Varies in IPD Patients

Next, we investigated potential alterations in the expression of CCN and ECCN elements in the IPD cohort. *DBP*, a clock gene and a member of the family of PAR BZIP transcription factors, was differentially expressed in the female patients versus the respective control group (p = 0.002, $\log_2$FC = 0.146, Figure S10). We could not find ECCN elements to be differentially expressed in the full cohort (Figure S11) nor in male patients (Figure S12). We additionally performed a correlation analysis for the CCN and the ECCN elements in the IPD cohort data sets. The determined Spearman correlation coefficient was compared with the reference correlation values for the CCN and ECCN genes in the control groups and visualized using correlation matrices ordered based on the pattern of the control male subjects, for comparison (Figure 6A–D). The IPD male and the female patients showed a weaker correlation for both the CCN and the ECCN elements, compared to the respective control groups, as indicated by the Spearman coefficients (Figure 6A–D). To avoid redundancy between elements of the CCN and of the ECCN gene list, only the genes which are not part of the CCN were visualized in the ECCN correlation heatmaps (Figure 6C,D). Within the CCN, one set of genes (*CLOCK, NR1D2, PER2, ARNTL, CRY1, RORA*) showed positive correlation within the group, and negative correlation with the other set of genes (*CRY2, RORC, NR1D1, NPAS2, PER1*) in the IPD and control group. For the ECCN, *CSNK1D, NFIL3, ALAS1, PARP1, CREBBP, BHLHE40, GSK3B, CSNK2A1, EP300, CREB1, PRKAA1, SIRT1, FBXL3, NONO,* and *PRKCA* showed the strongest positive correlation within the group, and weaker or negative correlation with the second set of genes (*PPARA, TEF, CSNK1E, WDR5, TNF, DBP*) in the IPD and in the control groups (Figure 6C,D). *BTRC* showed negative correlation with all other genes within the ECCN, both for IPD patients as well as for the control groups. Although the pattern of correlation was qualitatively similar among the different groups, there was a weaker correlation among ECCN elements as suggested by the overall decrease in the Spearman correlation coefficients for the cohort of IPD patients (both for males and females) compared to the respective controls, which points to a dysregulation of the circadian clock network associated with the disease. This data also highlights the need for investigating the role for the circadian clock in IPD by generating time-course data sets to monitor possible alterations in the circadian expression of relevant genes in IPD progression.

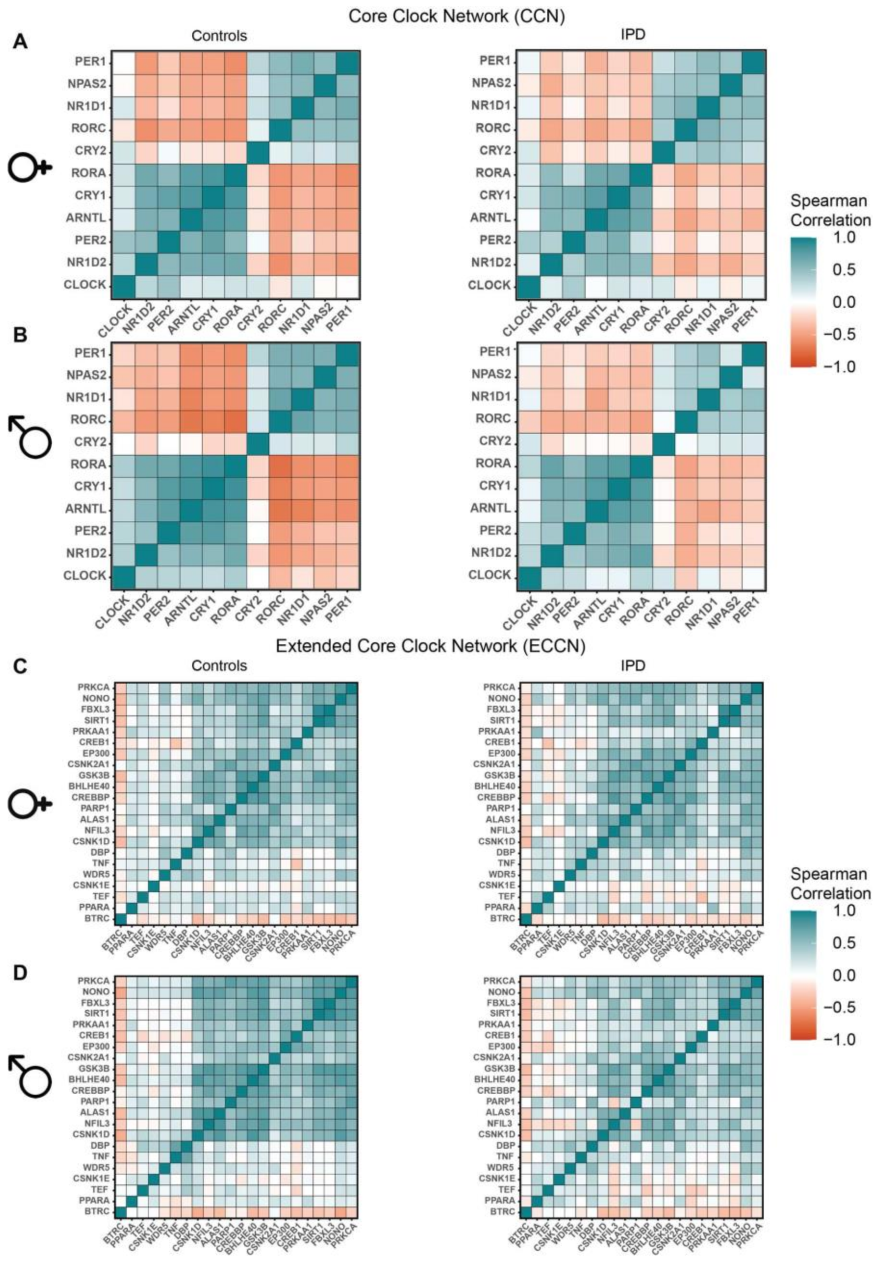

Figure 6. Correlation heatmaps of CCN and ECCN genes in IPD patients versus controls (sex separated). (**A**,**B**) Heatmap visualization of Spearman correlation between CCN genes in IPD, as well as the controls for (**A**) females and (**B**) males. (**C**,**D**) Heatmap visualization of Spearman correlation between each pair of the ECCN genes in IPD as well as in the control group for (**C**) females and (**D**) males.

3.4. Genes of Interest Exhibiting Differential Expression in Both CRC Cells and the IPD Cohort Show Alterations in Their Rhythmic Phenotype upon KO of Core-Clock Genes and Impact Survival in Colon Cancer Patients

To characterize the potential effect of clock dysregulation in the rhythmicity patterns of the top differentially expressed genes in the IPD cohort, we selected, out of the set of genes of interest, the ones present in the list of the top 100 differentially expressed genes (sorted according to *p*-value) in the full cohort (without sex separation), as well as in the top 100 differentially expressed genes (sorted according to *p*-value) in female and male patients. Because the IPD data set was collected at a single time point, we used the HCT116 time course data sets to visualize possible rhythmic variations in gene expression. Within the IPD female patients, *GBA*, a crucial regulator of lysosomal functioning, was found to be among the most common genetic factors in IPD patients [55,93] and was circadian expressed in HCT116^{WT} cells. Mutations of *GBA* gene are linked with increased IPD incidence. Upon clock disruption, *GBA* lost circadian rhythmicity in all KO conditions (Figure S13, middle panel). Among the top differentially expressed genes in the IPD male cohort, *CYBA* gene was circadian expressed only in the HCT116^{WT} condition, whereas in all KO conditions, its rhythmicity was lost (Figure S13, bottom panel). *CYBA* functions as a regulator of oxidative stress, phagocytosis and immunity, processes known to be disrupted in PD. In addition, we have analysed the circadian profiles of the PD-related gene subset within the genes of interest (Table S7) that also showed alterations in their rhythmicity patterns (loss or gain of oscillations or a change in amplitude or phases) upon disruption of core-clock genes in CRC cells (Figures 7A–C and S3). The genes showing an alternation in one of the parameters in either of the KO cells were visualized in all conditions in order to allow for a comparison between different core-clock KOs. *DRD1* showed an alteration in its rhythmicity in HCT116-*PER2*^{KO} (Figure 7B) and HCT116-*NR1D1*^{KO} (Figure 7C) compared to WT cells. *DRD1* was not expressed (based on our expression cutoff) in *ARNTL*^{KO} cells (Figure 7A). Among the PD-associated genes, which showed a fluctuation in their rhythmicity, *GBA* and *ADRM1* were differentially expressed in females and *TUBB2A* was differentially expressed in male IPD patients. These results suggest a potential role for the circadian clock as a regulator of genes involved in PD-associated molecular mechanisms. Altogether our results show that several of the differentially expressed genes found in the cohort of IPD patients are likely to show circadian variations and to be altered via perturbations of the core-clock, which may explain some of the molecular and physiological alterations reported in PD.

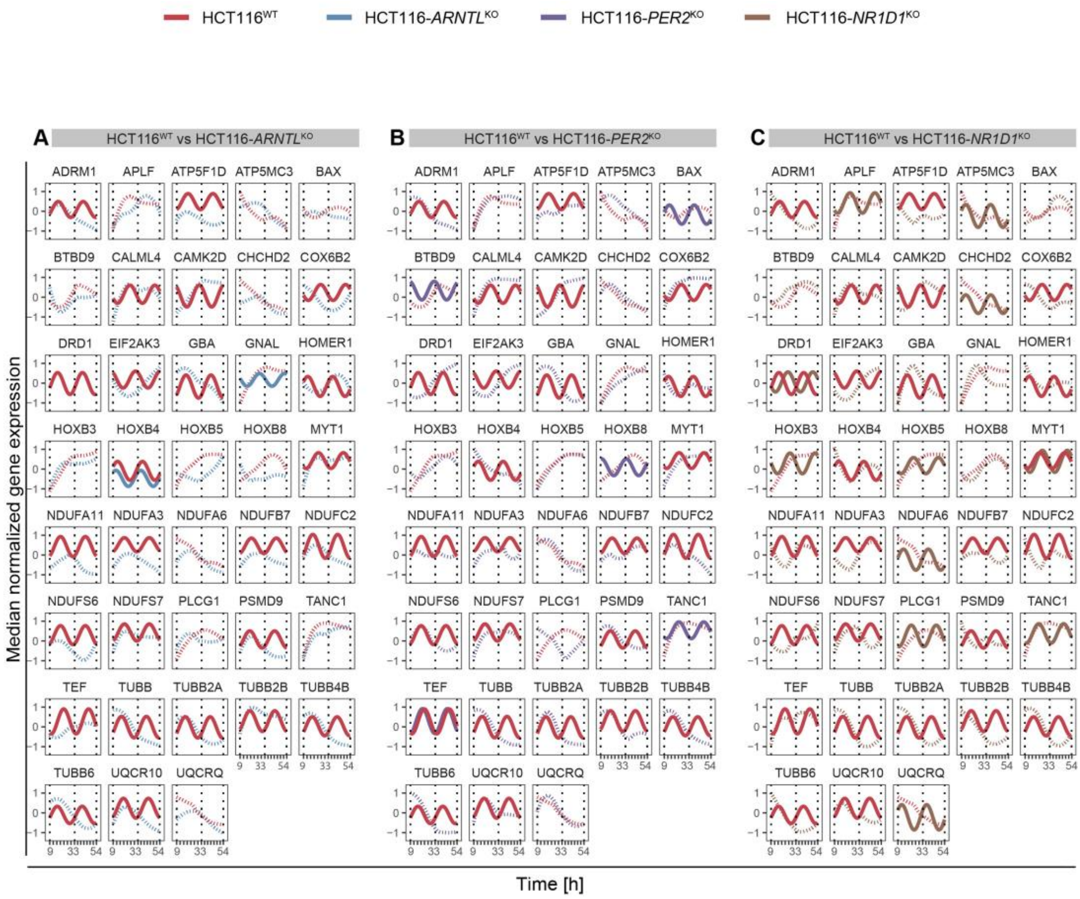

Figure 7. Circadian expression profiles of PD-associated genes. (**A**) in HCT116^{WT} and HCT116-*ARNTL*^{KO} (**B**) in HCT116^{WT} and HCT116-*PER2*^{KO} (**C**) in HCT116^{WT} and HCT116-*NR1D1*^{KO}. Rhythmicity analysis was computed considering a 24-h period. Depicted are significantly rhythmic genes ($q < 0.05$ and relative amplitude ≥ 0.1) plotted using a harmonic regression fitting (full lines), whereas not significantly rhythmic genes were represented with LOESS (dashed lines) (see Figure S3 for similar analysis in SW480 and SW620 cells).

To investigate the significance of our results in colon cancer, we merged all relevant genes resulting from our analysis (473 unique set of genes, Table S11) and intersected with the cancer hallmarks genes extracted from the CHG (Cancer Hallmark Genes) database [30]. To do so, we selected the genes from our analysis according to the following criteria: (i) Genes exhibiting a differential rhythmicity in at least one of the KO cells (compared to WT); (ii) Common genes exhibiting differential expression in at least one of the HCT116^{KO} cells (compared to WT) and in the IPD cohort (sex separated, and also the full cohort without sex separation); and (iii) top 100 differentially expressed genes in the IPD cohort (sex separated, and also the full cohort without sex separation compared to respective controls). Our analysis revealed that 188 genes (about 40% of the total number of 473 genes short-listed based on our analysis) were associated with cancer hallmarks. The largest number of these genes were linked to "resistance to cell death", followed by genes linked to "sustaining proliferative signaling" and "activation of invasion and metastasis" (Figure 8A).

Next, we performed a Kaplan–Meier survival analysis using TCGA COAD RNA-seq datasets, which revealed 289 genes out of the total number of genes found to be differentially expressed between cancer and normal tissue samples, to be significantly associated with patient survival. We found that four genes were also present in our analysis using the IPD cohort and CRC data sets, and were also part of the list of cancer-relevant genes retrieved from the CHG database [56]. These included the following: *TUBB6*, associated with 'activation of invasion and metastasis' and 'tumour promoting inflammation'; *PAK6*, linked to 'sustaining proliferative signaling', 'cell death resistance', 'activation of invasion and metastasis', 'evading growth suppressors' and 'immune destruction'; *SULT1A1*, linked to 'genomic instability and mutation', and *SLC11A1*, associated to 'resistance to cell death' (Figure 8B). *TUBB6*, which encodes for microtubules and has a crucial role in mitosis, intracellular transport and neuronal communication (motility and morphology), lost circadian rhythmicity in all KO cells (compared to WT). Downregulation of *TUBB6* expression resulted in increased survival probability in the TCGA-COAD cohort (Figure 8B). The differential expression of *PAK6*, which encodes for a member of p21-stimulated protein kinase family functioning in apoptosis and cytoskeletal rearrangement, was found to be significantly associated to patient survival in TCGA-COAD data and differentially expressed in $ARNTL^{KO}$ and $NR1D1^{KO}$ HCT116 cells, and in the IPD female patient cohort (Figure 8B). Downregulation of *PAK6* resulted in increased overall survival compared to high expression in the TCGA-COAD cohort. Among the top 100 differentially expressed genes in male IPD patients, *SULT1A1* encoding for a member of sulfotransferase enzyme family that catalyzes several hormones, neurotransmitters, as well as drug compounds and *SLC11A1* encoding for transporters involved in iron metabolism were found to be relevant for overall patient survival. Downregulation of *SULT1A1* resulted in a lower survival probability, whereas lower expression of *SLC11A1* yielded a higher survival probability in the TCGA-COAD cohort. Of note, additional clinical traits could be classified as a risk factor (e.g., age, sex, smoking, treatment) and may influence the outcome of survival analysis. While making an accurate assessment of all clinical traits remains a challenging task for the analysis of the data, we tried to address this issue by performing a comparative analysis using age, sex and disease stage as stratification factors and investigated the putative impact of these traits on patient overall survival. Among the clinical variables that we analyzed in the TCGA-COAD dataset, neither age at diagnosis nor sex were found to be significantly associated with survival (Figure S14A–D). Yet, cancer stage, was associated significantly with survival when the cohort was separated into four subgroups (Stage 1, 2, 3, 4) (Figure S14C). However, when subgrouping the cohort into early (1 and 2) and late (3 and 4) stage groups, we did not observe a significant impact of stage on the survival analysis (Figure S14). To further investigate the influence of the pre-selected differentially expressed genes on survival when considering cancer stage, we used a Cox Regression model to assess stage as a stratification factor for adjustment of the statistical testing (Table S12). Among the previously found cancer-hallmark survival-related genes (*PAK6*, *SLC11A1*, *SULT1A1* and *TUBB6*), we observed that their differential expression still had a significant impact on survival for three of the genes (*PAK6*, *SLC11A1* and *TUBB6*), when considering stage stratification. In addition, we identified four additional genes that were not included in the CHG database, but resulted from our analysis using IPD and CRC data sets and that showed a significant association with overall patient survival (Figure S15). These included *TRAP1*, which encodes for a molecular chaperone member of Hsp90 (Heat Shock Protein) family and was commonly differentially expressed in all $HCT116^{KO}$ cells and in IPD male cohort; *FAM50B*, encoding for a protein associated with chromatin organization; *SPON2*, involved in cellular adhesion and embryonic neuronal outgrowth; *FES*, encoding for a tyrosine kinase that is essential for the maintenance of cellular transformation, were among the top 100 differentially expressed genes in the IPD cohort (without sex separation) and found to be significantly associated with survival in cancer patients (Figure S15). Interestingly, *FAM50B* was downregulated in HCT116-$ARNTL^{KO}$, HCT116-$NR1D1^{KO}$ and in male IPD patients, whereas it was upregulated in

HCT116-*PER2*^{KO} cells, which points to an alteration in the expression of this gene both in IPD and as a result of core-clock modifications. Downregulation of *FAM50B*, *FES* and *SPON2* resulted in an increased survival probability, whereas upregulation of *TRAP1* was associated with higher survival probability. Altogether these findings suggest that several genes resulting from our analysis, altered in both CRC cells and IPD patients, are part of the cancer hallmarks gene sets and impact survival in colon cancer patients.

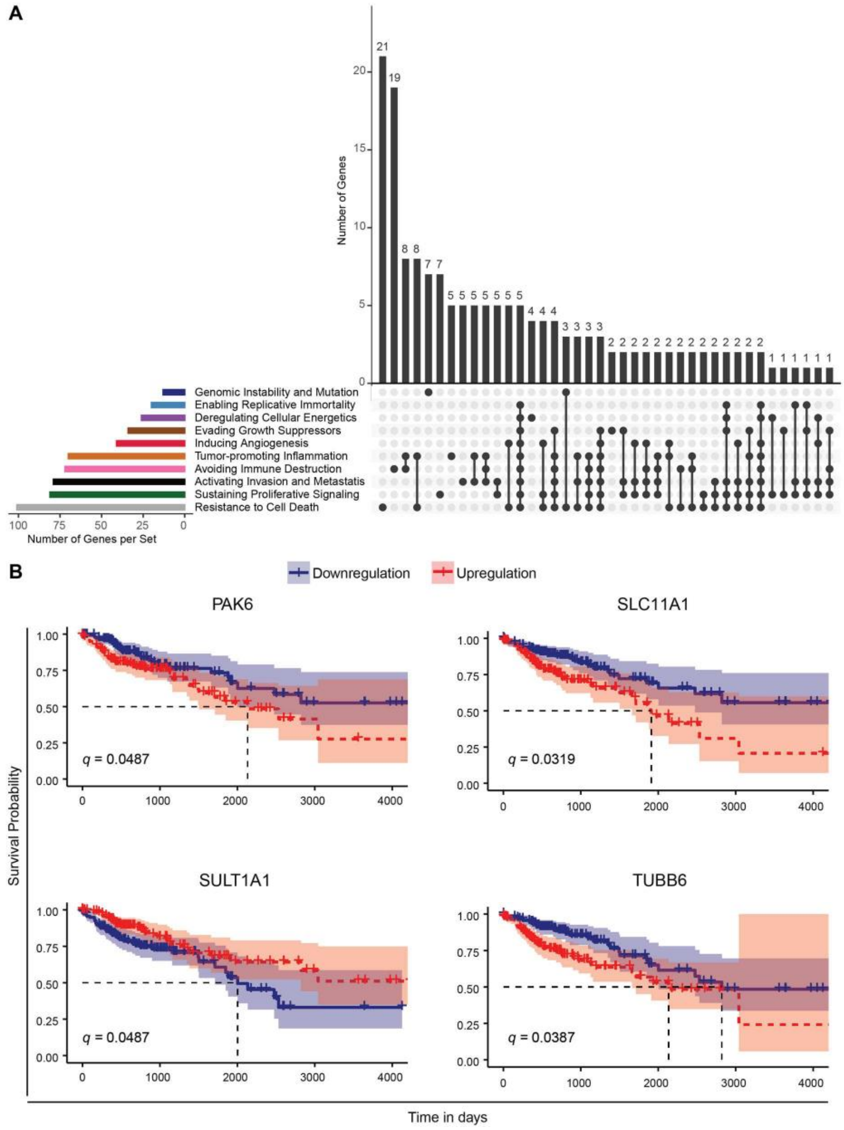

Figure 8. Correlation of genes resulting from the analysis with CRC cell lines and the cohort of IPD patients, with cancer hallmarks and survival in a colon adenocarcinoma cohort. (**A**) The plot depicts the intersection of cancer-hallmarks-related genes (retrieved from the CHG database [56]) and relevant genes highlighted by our analysis (Table S11). The colour coded bar plots (**left**) indicate the number of genes related to a particular cancer hallmark. The genes found to be associated with more than one hallmark are indicated with connecting lines (**right**). The number of unique genes and genes associated with

more than one cancer hallmark are indicated above the grey bar plots (**right**). (**B**) Kaplan–Meier survival curves for genes found to be common in our analysis and genes associated with patient survival in the TCGA-COAD cohort. The patient cohort was separated into two groups based on the median gene expression level as high and low expression groups and a comparison of survival time (in days) was performed. In a subsequent analysis, using cancer stage as stratification of the patients, the following q-values were obtained: PAK6, $q = 0.036$; SLC11A1, $q = 0.005$; SULT1A1, $q = 0.27$; TUBB6, $q = 0.01$ (see also Table S12).

4. Discussion

PD is an intrinsically fluctuating disorder. As the disease progresses, patients describe symptomatic daily variations [94], commonly attributed to the administration timing of anti-parkinsonian drugs. Patients suffer from disabling off periods, as the dopaminergic drugs fade away, or, conversely, troublesome dyskinesias arise in synchrony to peak levels of these same drugs [95]. There are, however, fluctuations that are unrelated to drugs, and empirical clinical evidence suggests that some fluctuations follow a 24-h rhythm. As the disease progresses, patients report a progressive decrease in motor function late in the day [96,97], also known as "sundown syndrome", and in more advanced PD stages, there is a common complaint of night-time akinesia, which affects over 70% of patients and prevents patients from changing sleep position or easily moving out of bed [98]. Contrary to this, some patients report an improvement in motor-, cognitive- and mood-related symptoms after waking up, even though they did not take regular medications during the night [99]. The motor response to repeated L-dopa administration also shows fluctuations during the day with weaker responses late in the evening and afternoon, as compared to the morning doses [100]. Visual discrimination deficits, which are frequently affected in PD, are also more prominent in evening hours [101]. Clinically, PD patients show blunted amplitudes in physiological variables that are expected to show diurnal variations. The rest-activity cycle measured through actigraphy shows decreased activity during the day in PD patients and higher movement levels during the night, resulting in an overall flattening of the circadian curve [102–106]. Studies on the variation of autonomic function throughout the day also suggest circadian changes in PD patients related to core body temperature profile [107–109], blood pressure [110] and heart rate variability [111,112]. Aziz et al. [113] evaluated the secretion profiles of growth hormones, thyroid-stimulating hormone, prolactin, leptin, adiponectin and resistin in de novo untreated PD patients and found no alterations. Cortisol, on the contrary, has also been shown to have an elevated secretion profile during the night in PD [50,114]. The dosage and timing of dopaminergic medications has been shown to increase melatonin secretion, another hormone with known circadian variations [115,116]. Melatonin, one of the important components of the circadian system, was shown to have a 24-h secretion profile significantly lower in PD patients, in a controlled design study using a modified constant routine [117] and in another sample of early PD patients [50]. Moreover, individual differences in melatonin profiles have been reported including a marked variability that occurs in PD in contrast to controls [116,118]. It is also possible that melatonin changes are not ubiquitous in PD because it is increasingly recognized that there are different subtypes of this disease [119]. In addition, sleep disturbances, one of the core clinical features of circadian dysfunction, are very common in PD, with different clinical manifestations ranging from insomnia to excessive daytime sleepiness [20,120,121]. The link between these observations from clinical data, physiological and molecular changes and circadian dysfunction is tempting, despite still-incomplete evidence.

4.1. Circadian Dysregulation of Cancer and PD-Related Pathways

Cancer development has also been linked with circadian dysregulation with several studies pointing to the role of core-clock genes as tumour suppressors [28,34,35,122,123]. Thus, and even though these two pathologies seem rather opposite in terms of cellular fate output [3,86], PD and cancer are both age-related diseases that share common disrupted pathways. According to our results, these shared altered pathways are likely circadian regulated. These commonly altered mechanisms include malfunctions in several processes including redox homeostasis, functioning of immune system, cell cycle, DNA repair and circadian regulation [3,6,13,14]. Our analysis shows that upon perturbation of core-clock genes, the rhythmicity pattern of genes involved in such processes was altered. According to our findings, the KO of *PER2*, which has previously been reported to act as a tumour-suppressor (reviewed in [124]), led to a drastic reduction in the number of oscillating genes compared to HCT116^{WT} cells and the strongest change in the phase distribution in all investigated periods. Differential rhythmicity analysis reflected loss of rhythmicity of lysosomal biogenesis and autophagy genes, which are known to be frequently disrupted in PD [125,126]. This subset of genes included *TFE3* and *MCOLN2* (altered in *PER2*^{KO} cells), *NAGLU* (altered in *ARNTL*^{KO} cells) and *PP2CA* and *ACP2* (altered in *NR1D1*^{KO} cells). *PPP2CA* encodes for the catalytic unit of phosphatase 2A (*PP2A*), which acts as a tumour suppressor [127]. It prevents WEE1 (G2/M checkpoint regulator) from proteolysis, thereby leading to elevated levels of WEE1 protein and subsequent cell cycle arrest, and thus results in an impairment of cellular proliferation (reviewed in [128]). PP2A was found to be down-regulated in certain types of cancer (such as glioblastoma [129]) and neurodegenerative disorders including Alzheimer's disease [130] and PD [131]. In PD, low activity of PP2A has been reported, and α-synuclein, a key component in the pathology of PD due to its abnormal accumulation, which results in Lewy bodies, acts as a regulator of PP2A. Based on our study, the abolished rhythmicity of *PP2CA* in *NR1D1*^{KO} can potentially suggest that its circadian variation might be regulated by *NR1D1*. A previous study in drosophila reported that PP2A regulates PER phosphorylation, thereby governing its activity in vitro and in vivo [132]. However, to the best of our knowledge, no studies have previously suggested a potential association between PP2A and the circadian clock in mammals. *NOS3*, an inhibitor of apoptosis and promoter of angiogenesis, lost circadian expression in all KOs compared to WT cells. *NOS3* was shown to play a pivotal role in cancer by promoting angiogenesis, metastasis formation and invasiveness, inhibition of apoptosis and immune response. Among metastatic colorectal cancer patients, *NOS3* inversely correlated with disease-progression-free survival and overall survival [133]. Interestingly, *NOS* isoforms (*NOS1*, *NOS2A*, and *NOS3*) are suggested to be strong candidates as susceptibility genes linked to environmental stress in PD [134]. Another important example of circadian regulated genes in both cancer and PD is *GADD45A*, a downstream mediator of p53, and regulator of the cell cycle and DNA repair. *GADD45A* acts as a tumour suppressor [135] and was found to be correlated with the expression of the core-clock gene *PER2*, in human colorectal carcinoma tissues [136]. In addition, in an in vitro model of PD, the knockdown of *GADD45A* was proposed to be neuroprotective against neuronal death induced by MPP (1-Methyl-4-phenylpyridinium), which is a metabolite used to mimic mitochondrial impairment in in vitro models of PD by inducing dopaminergic neuronal death [137]. Our results showed that *GADD45A* was differentially rhythmic in *NR1D1*^{KO} cells, as well as in SW480 versus SW620 cell lines, where we observed circadian rhythms of *GADD45A* only in the SW480 cells. Taken together, the variations in the circadian phenotypes of these genes upon core-clock perturbation point to a role for circadian regulation in genes which are involved in PD and cancer.

Mitochondria, the key component of energy metabolism, is tightly linked to several high ATP demanding neuronal processes such as axonal transport, due to its role in ATP production. Elevated lipid peroxide levels and mitochondrial malfunctioning has been previously shown in PD [138,139]. Strikingly, we observed a loss of rhythmicity of mitochondrial and oxidative phosphorylation genes of the NDUF family in our KO cell lines (*NDUFA11* in all KO conditions, *NDUFS7*, *NDUFC2* in *NR1D1*^{KO} cells and *NDUFS6* in *ARNTL*^{KO} cells). In addition, several clock genes were differentially rhythmic in the KO cells (*CRY1*, *NR1D1*, *DBP*, *TEF*, *BHLHE41*). Interestingly, *TEF* has been previously linked to a *TEF* variant associated with PD (rs738499) and found to be associated with sleep disturbances in PD [47]. Furthermore, the dopaminergic receptor *DRD1* was found to be differentially rhythmic in *NR1D1*^{KO} cells, and showed a phase delay compared to

WT cells. Aberrant interactions between different dopamine receptors in the brain may be involved in L-dopa-induced dyskinesia [140] and risk of hallucination [141], and based on our findings circadian factors might also be involved in this interplay. Moreover, a regulator of vesicle trafficking, *LRRK2*, which is a frequently mutated gene in PD [142,143], has been shown to impair *DRD1* signalling transduction in mice [144].

4.2. Clock-Controlled Genes Are Differentially Expressed in PD

Our analysis indicated that some of the differentially rhythmic genes in the CRC data were also differentially expressed in the IPD cohort. These included genes linked to oxidative stress and energy metabolism such as *CYBA*, *TFE3*, *DBP* and *GBA*. *GBA* encodes for the lysosomal enzyme β-glucocerebrosidase and mutations in *GBA* are a key risk factor identified for patients with IPD [93,145,146]. *GBA* was upregulated in *ARNTL*^{KO} cells and *NR1D1*^{KO} cells, as well as in the IPD patients. Moreover, *GBA* was rhythmic in HCT116^{WT} cells, whereas its rhythmic expression was lost in all KOs. PD patients carrying *GBA* mutations show more dyskinesia, as well as other fluctuating symptoms like dysautonomia and hallucinations [147]. We observed that changes on the mean gene expression level of PD patients resembled similar trends (up/downregulation) within the set of genes of interest, as in KO cells. This synergistic effect was the case for the cell cycle regulator *CDKN1C* (upregulated in all KO cells and IPD data sets), and genes related to the immune system such as *TLR6* (upregulated in HCT116-*PER2*^{KO} and IPD data sets), *SERPINA1* (upregulated in all KO cells and the IPD data sets) and the PD-associated gene *GBA* (involved in energy metabolism), synergistically upregulated in IPD data sets and HCT116-*ARNTL*^{KO}, HCT116-*NR1D1*^{KO}. *TGFB1* involved in cell proliferation, inflammation and differentiation exhibited a synergistic effect in HCT116-*PER2*^{KO} and HCT116-*NR1D1*^{KO}, but an antagonistic effect in HCT116-*ARNTL*^{KO}, as compared to the IPD data sets. This points to a core-clock-specific effect on gene expression changes and highlights the fact that the regulation of these differentially expressed genes (common between HCT116 cells and IPD samples) might affect both diseases (cancer and neurodegeneration) in a different manner.

Another interesting example is the gene *SNCA*, which encodes for the protein α-synuclein. This protein was found to be accumulated in patients with PD and linked to Lewy bodies. Mutations in *SNCA* have been reported in PD patients with a genetic background [148] and elevated levels of *SNCA* were found in IPD [149] and shown to vary during the course of disease progression [150]. Moreover, *SNCA* mutations are responsible for a rare autosomal dominant form of PD that shows severe fluctuations and prominent psychiatric features including hallucinations and autonomic dysfunction [147]. Our results showed upregulation of *SNCA* only in *ARNTL*^{KO} cells, whereas it was downregulated in *NR1D1*^{KO} cells, as well as in IPD patients. This suggests that perturbation of core-clock genes may result in differential outputs in *SNCA* expression levels. The specific clinical profile of patients with either *GBA* or *SNCA* mutations with prominent motor and nonmotor fluctuations and the results from our analysis provides a putative link between circadian dysfunction and these important clinical symptoms.

Our results further point to sex-specific variations in gene expression within the PD cohort. Female patients showed more genes to have expression changes in the same direction as in the clock KO cells, as compared to male patients. *DBP*, a clock gene, was differentially expressed uniquely in females. Its rhythmic pattern was abolished in *ARNTL*^{KO} cells and upregulated in *PER2*^{KO} and *NR1D1*^{KO} cells. Previous studies have shown the loss of circadian expression of *Dbp* in *Arntl* KO mice, and manipulation of *Arntl* was found to be tightly linked to neurodegeneration [151]. Furthermore, a study investigating changes in colonic mucosa of healthy and neoplasmic mice suggested reduction in amplitudes and phase delay in circadian expression of *DBP* in colonic mucosa of healthy mice compared to colonic neoplasm [152], thus strengthening the potential role of the circadian clock in the crosstalk of the two pathologies. Even though no additional clock gene was differentially expressed in the cohort of IPD patients, our data showed a weaker correlation of CCN and

ECCN genes in IPD when compared to controls (sex separated), pointing to a change in the relative expression of clock genes due to PD. The expression of clock genes has been shown to be altered in PD, as already discussed [22,48,50]. The overall lack of available time series data in the cohort of IPD patients and the lack of information regarding the timing of blood sampling in the IPD dataset analyzed did not allow us to carry out a circadian analysis of the data sets. Nevertheless, to overcome this limitation, we have performed differential expression analysis using both the CRC and IPD data sets, and we have additionally analysed circadian profiles of our genes of interest among the top 100 differentially IPD expressed genes in our CRC data. Out of these genes, we observed a gain of oscillations in *DAPK2* (in *ARNTL*^{KO} cells), *ATP6V0D1* (in *NR1D1*^{KO} cells) and *CXCL16* (in *PER2*^{KO} cells) compared to HCT116^{WT} cells, whereas a loss of rhythmicity was observed for *GBA* and *CYBA* for all KO conditions. Taken together, our study shows that IPD patients and core-clock KO CRC cells resemble similar trends in alteration of gene expression for the pre-defined genes of interest and suggest a role for the circadian clock in the regulation of molecular pathways that are altered in cancer and neurodegeneration. However, the generation of time-course data sets for PD patients in future studies is needed and may allow for a better identification of molecular signatures for prognostic and diagnostic purposes in PD, by also considering the circadian profile of disease-associated genes.

Our analysis pointed to a link between the genes that resulted from the time-series analysis in CRC cells and the differential expressed genes in the IPD datasets, and cancer hallmarks genes retrieved from CHG database [56]. Namely, we found associations to "resistance to cell death", "sustaining proliferative signaling" and "activation of invasion and metastasis", which reinforces our initial hypothesis of a correlation regarding common altered pathways both in cancer and neurodegeneration.

Furthermore, a subsequent analysis on the possible role of the altered genes in CRC and IPD data sets in the survival of cancer patients (TCGA-COAD data sets) pointed to a significant influence on survival for eight of these genes (*TUBB6*, *PAK6*, *SULT1A1*, *SLC11A1*, *TRAP1*, *FAM50B*, *SPON2*, *FES*). Our results showed that downregulation of *PAK6* resulted in a higher overall survival probability in colon cancer patients. In line with our observation, *PAK6* was reported to be associated with overall survival, enhancing chemotherapeutic resistance and was proposed as a prognostic marker for colon cancer patients [153]. Furthermore, it was shown that *PAK6* interacts with *LRRK2* whose mutation is linked to a genetic form of PD and its aberrant activation was observed in IPD [154–156]. Interestingly, *PAK6* was upregulated in *ARNTL*^{KO}, *NR1D1*^{KO} cells and IPD female patients, suggesting a potential function for *PAK6* linking cancer and neurodegeneration. Another significant example is *TUBB6* whose downregulation yielded a better survival probability in TCGA-COAD cohort and was found to lose rhythmicity in all KOs, in our analysis. *TUBB6* was previously proposed as a prognostic biomarker in several cancer types including gastric [157], ovarian [158] and prostate cancer [159]. Furthermore, *TUBB6* was found to be associated with inflammatory induced cell death response [160] and reported to be also a direct interactor with *LRRK2* kinase [161], which is involved in PD [144,156], as described above. The loss of rhythmic expression of *TUBB6* points to a regulation of this gene via the circadian clock. These findings suggest that several of the genes highlighted in our study are likely relevant in a colorectal cancer and IPD context, impact the survival of cancer patients and highlight potential novel links between cancer and neurodegeneration. Future experiments will be needed to further validate and investigate the exact role of circadian regulation (or the lack of it) of these genes in the context of different cancer types and PD. It is relevant to point out that even though we have carried out a survival analysis by also accounting for tumour stage, which highlighted this trait as a relevant factor contributing to survival, additional clinical traits, such as treatment regime, may also contribute to the prognostic potential of these candidates.

5. Conclusions

Empirical evidence of circadian dysfunction in PD is only beginning to be unveiled. Many studies from human subjects show conflicting results or rely on findings that are intrinsically multifactorial in origin. Further research requires the generation of time course data sets for PD patients and should focus on describing in greater detail the mechanisms underlying circadian dysfunction, its association and causative role in many of the clinical and physiological changes detected in these patients. Care should be taken when evaluating the circadian system in PD patients due to the possible reciprocal influence of neuropathophysiological variability and the circadian system. It is also important to point out that considering patient stratification based on specific clinical traits, prior to the analysis, may influence the results presented in this study, as shown by our analysis regarding the significance of factors like age, sex, and stage. A potentially relevant, but mostly neglected, clinical trait is the patients' circadian rhythm, which should be considered in future studies, and for which new methods for its accurate assessment and monitoring need to be further developed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13235978/s1. Figure S1: Heatmap for ECCN genes in HCT116 cells, Figure S2: Biological processes associated with 12-h rhythmic genes in HCT116 cells, Figure S3: Circadian expression profiles of genes of interest in SW480 and SW620 cells, Figure S4: Biological processes associated with differentially expressed genes in HCT116 cells, Figure S5: Biological processes associated with differentially expressed genes in IPD datasets, Figure S6: Heatmap for differentially expressed genes of interest in HCT116 cells, Figure S7: Commonly differentially expressed genes of interest in HCT116 KOs and males in IPD cohort, Figure S8: Commonly differentially expressed genes of interest in HCT116 KOs and in female and male IPD patients, Figure S9: Heatmap of top 100 differentially expressed genes (sorted by p-value) in males, Figure S10: Expression patterns of ECCN genes in IPD female patients, Figure S11: Expression patterns of ECCN genes in IPD patients, Figure S12: Expression patterns of ECCN genes in IPD male patients, Figure S13: Circadian expression profiles of genes of interest among the top 100 differentially expressed genes in the IPD cohort (without sex separation, top panel), in females (middle panel) and in males (bottom panel), Figure S14: Significance analysis on the impact of clinical traits potentially relevant for patient survival, Figure S15: Survival curves for short-listed genes associated with colon cancer patient survival, Table S1: Demographic information of GSE99039 dataset downloaded from GEO (date of data retrieval: 16.02.2021) and ArrayQualityMetrics outlier test results (failed tests are marked with "x"), Table S2: List of genes pre-selected from KEGG pathways in immune system module, Table S3: List of genes pre-selected from KEGG pathways DNA replication and repair, Table S4: List of genes pre-selected from KEGG pathways involved in cellular growth, Table S5: List of genes pre-selected from KEGG pathways involved in redox homeostasis (energy metabolism and catabolism), Table S6: List of genes from the ECCN, Table S7: List of genes pre-selected from KEGG pathways involved in PD and genes involved in PD-associated disorders restless legs syndrome (RLS) with possible circadian influences, Table S8: GO Biological Process Enrichment results for 24-h and 12-h rhythmic genes, Table S9: GO Biological Process Enrichment results for differentially expressed genes in HCT116 cells, Table S10: GO Biological Process Enrichment results for differentially expressed genes in the IPD Dataset, Table S11: List of genes from the analysis with CRC and IPD data sets, Table S12: The list of differentially expressed genes found to be associated with overall survival of TCGA-COAD patients and results from the Cox Regression analysis based on stratification for tumour stage.

Author Contributions: Conceptualization, A.R.; methodology, M.Y., D.M., A.B.; formal analysis, M.Y., D.M.; investigation, M.Y., D.M., A.B., A.R.P., J.J.F., A.R.; writing—original draft preparation, M.Y., A.R.; writing—review and editing, M.Y., D.M., A.B., A.R.P., J.J.F., A.R.; visualization, M.Y., D.M.; supervision, J.J.F., A.R.; funding acquisition, M.Y., A.R. All authors have read and agreed to the published version of the manuscript.

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Supplemantary Materials

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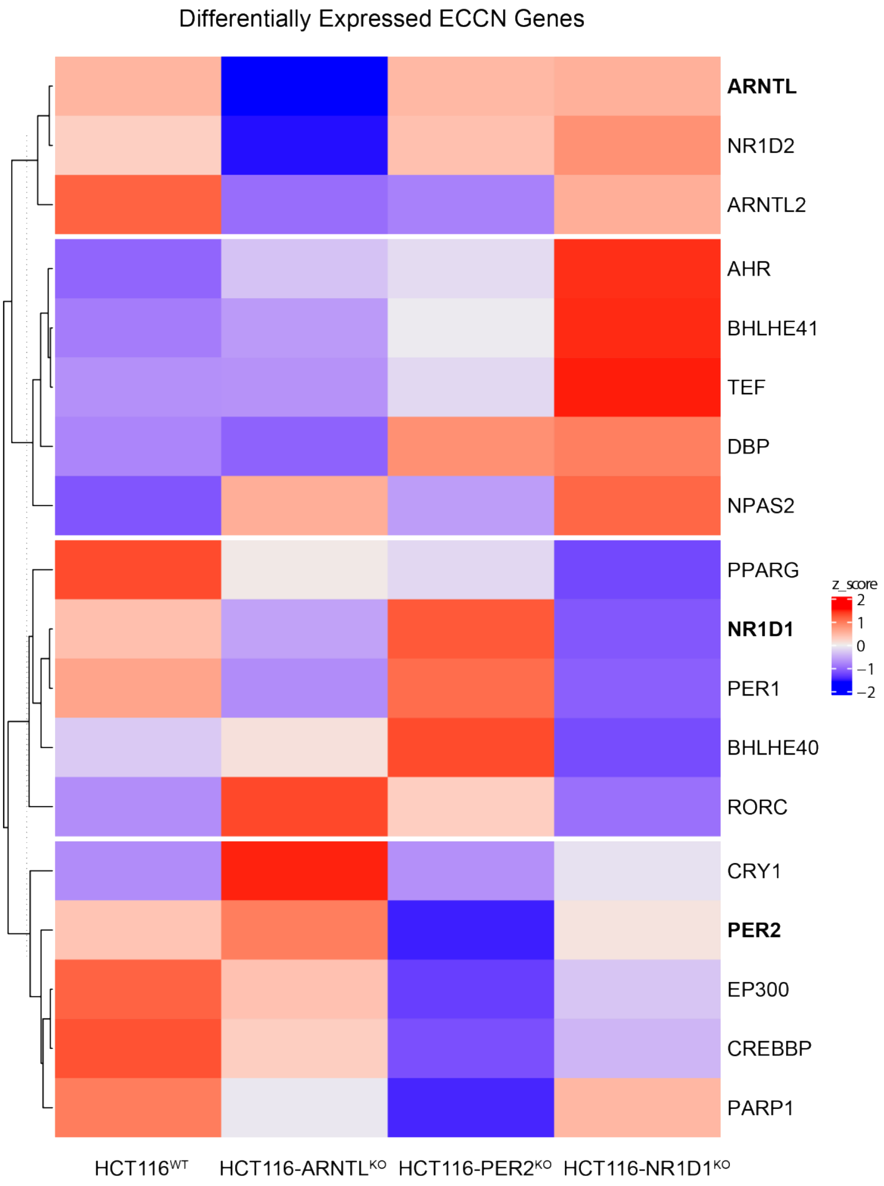

Figure S1. Heatmap of ECCN genes in HCT116 cells. ECCN genes that were differentially expressed in at least one of the Knock Out (KO) cells compared to HCT116^{WT} cells are depicted in the heatmap. KO genes (ARNTL, PER2 and NR1D1) are depicted in bold. Clustering was performed using k-means clustering (k=4). Colour code scheme for standardized gene expression values (z-scores) are indicated in the heatmap colour key.

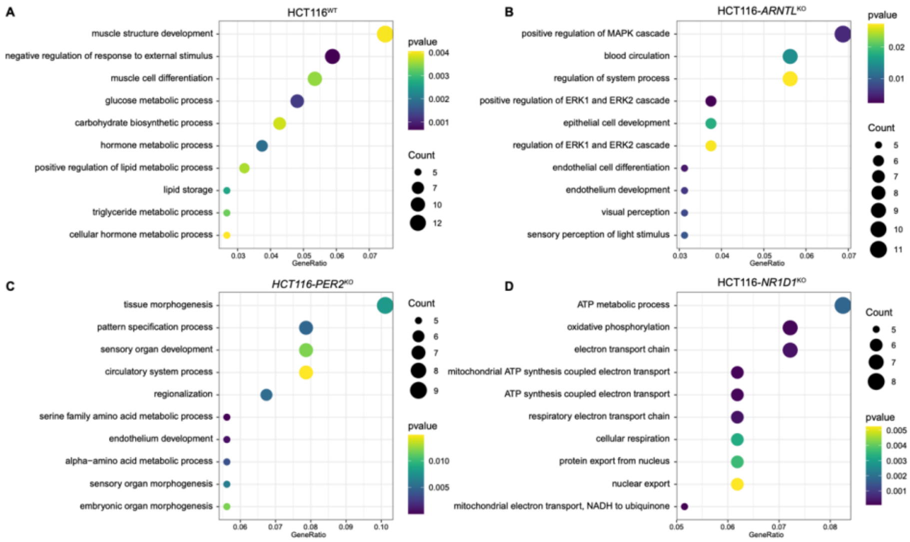

Figure S2. Biological processes associated with 12h rhythmic genes in HCT116 cells. *Top 10 GO (Biological Processes) enriched in* ***(A)*** *HCT116^{WT} cells,* ***(B)*** *HCT116-ARNTL^{KO} cells* ***(C)*** *HCT116-PER2^{KO}cells* ***(D)*** *HCT116-NR1D1^{KO} cells.*

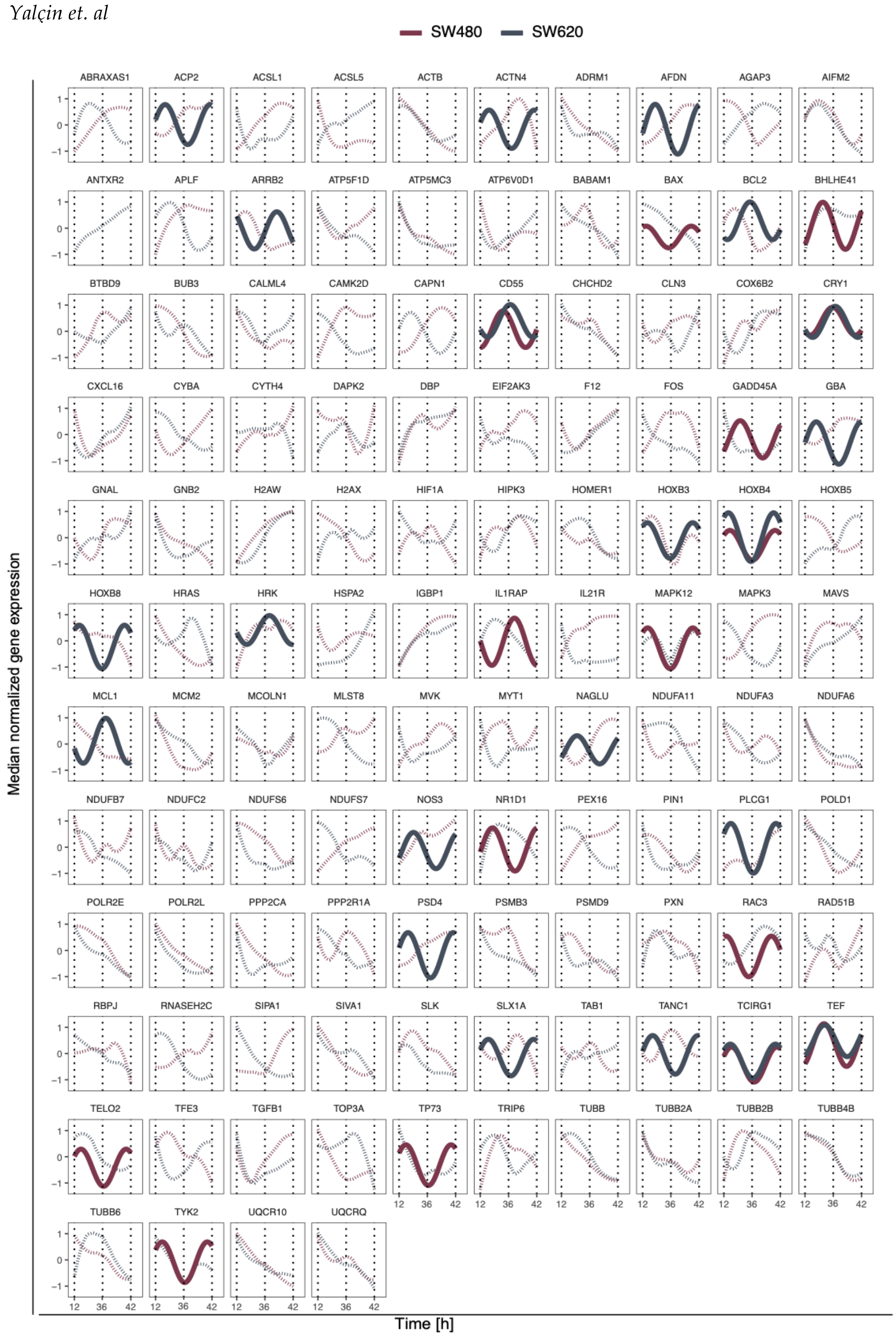

Figure S3**. **Circadian expression profiles of genes of interest in SW480 and SW620 cells. *Rhythmicity analysis was computed considering 24h-period. Genes that were found to be significantly rhythmic (q<0.05, relative amplitude ≥ 0.1) plotted using a harmonic regression curve (full lines) and non-significant ones are shown with loess smoothened lines (dashed lines).*

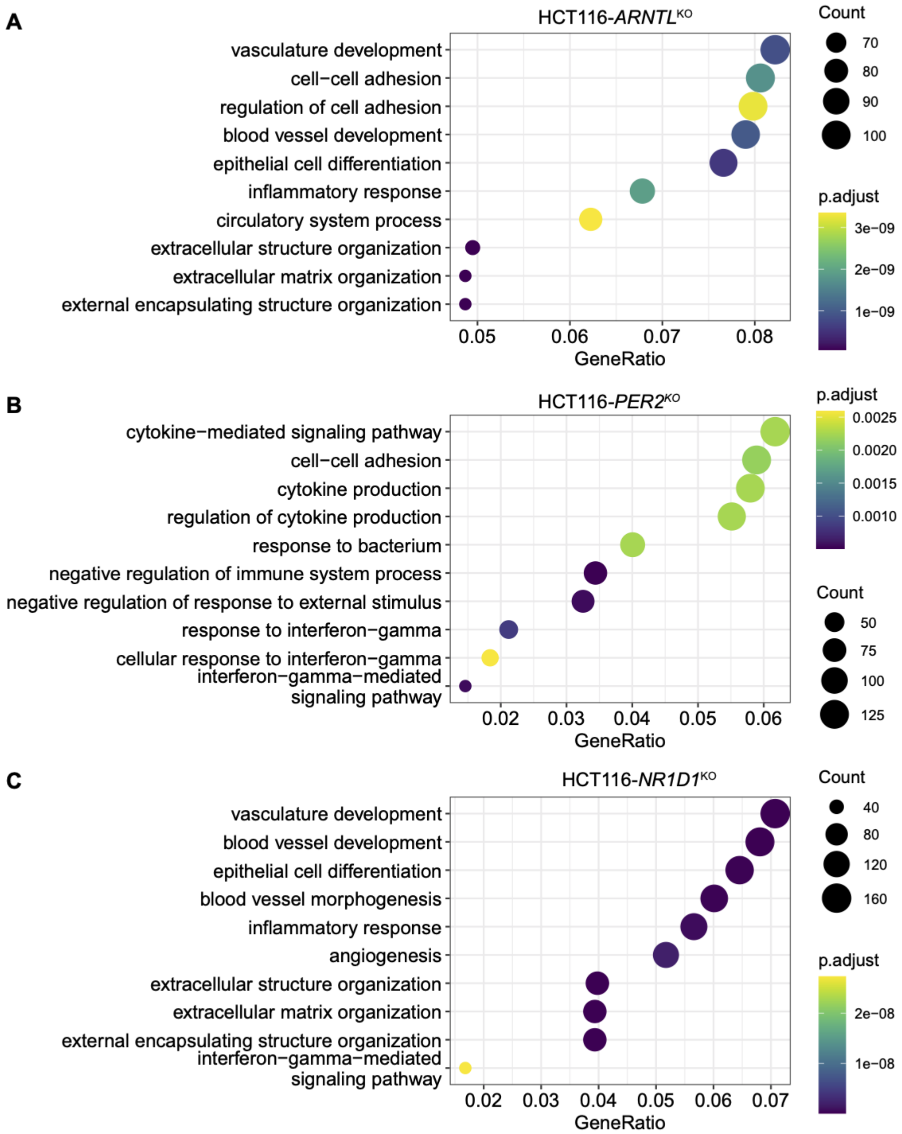

Figure S4. Biological processes associated with differentially expressed genes in HCT116 cells. *Top 10 GO (Biological Processes) enriched in **(A)** HCT116-ARNTL^{KO} cells vs HCT116^{WT} cells **(B)** HCT116-PER2^{KO} cells vs HCT116^{WT} cells **(C)** HCT116-NR1D1^{KO} cells vs HCT116^{WT} cells.*

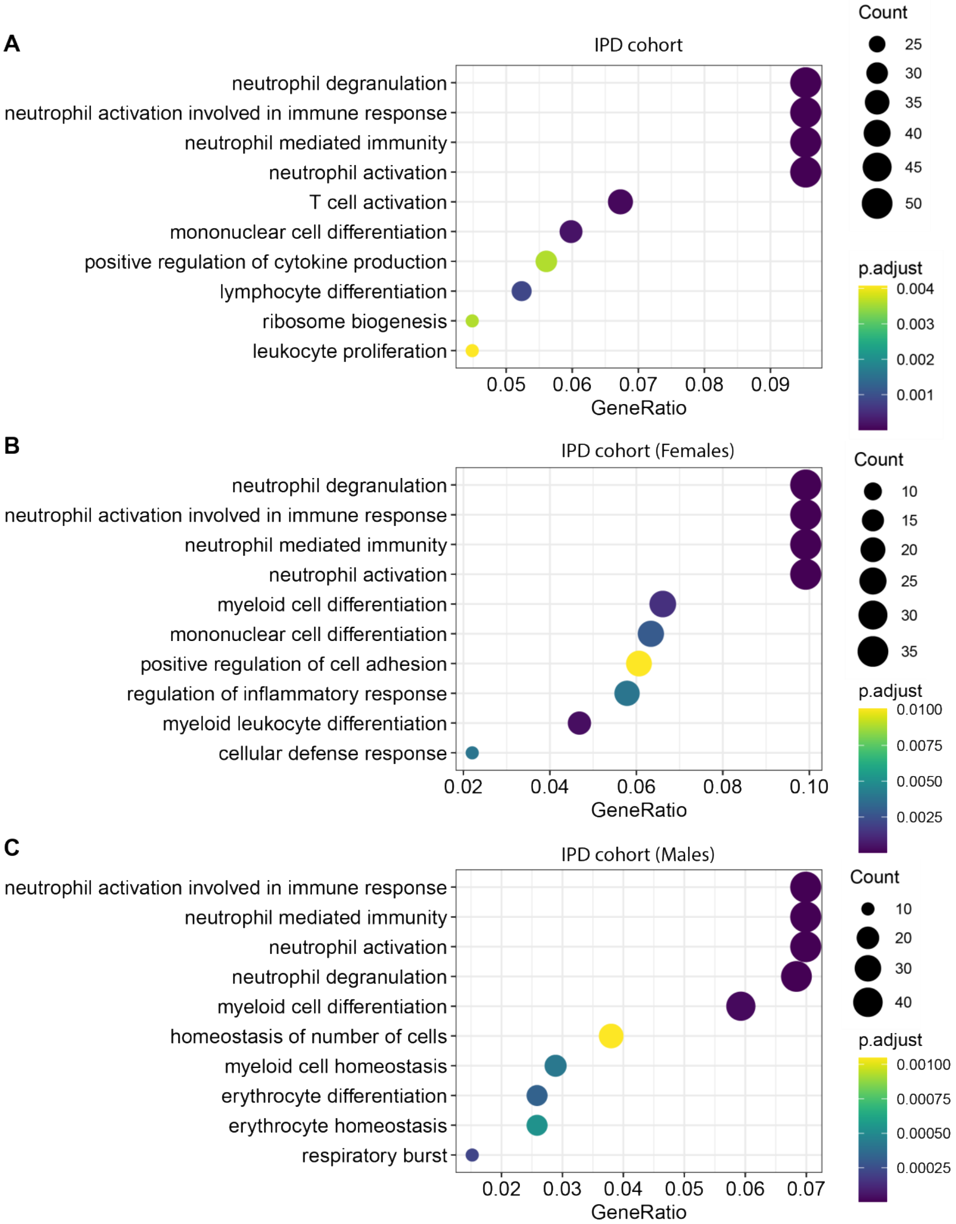

Figure S5. Biological processes associated with differentially expressed genes in Idiopathic Parkinson's Disease datasets. Top 10 GO (Biological Processes) enriched in (A) IPD patients vs respective controls, (B) Female IPD patients vs respective controls, (C) Male IPD patients vs respective controls.

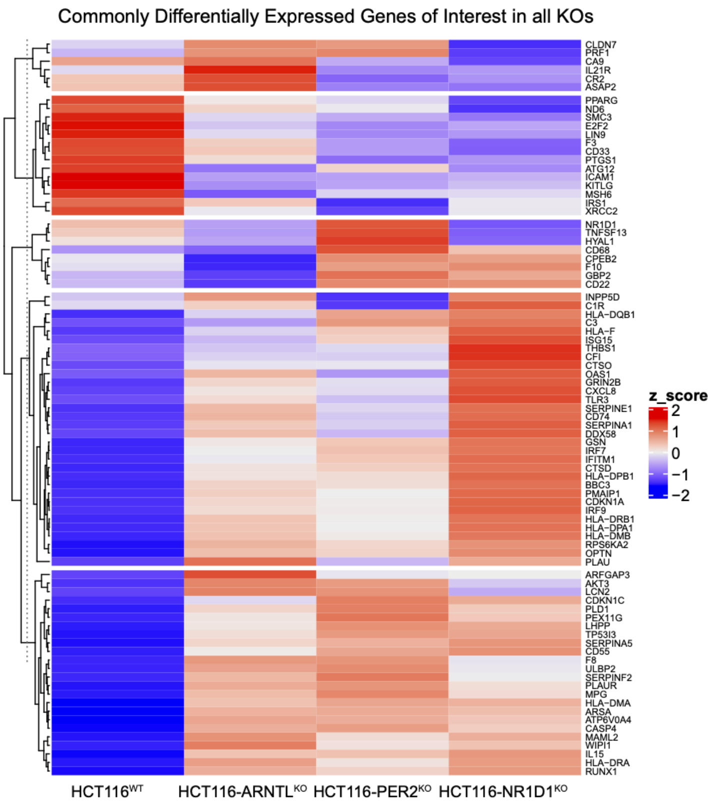

Figure S6. Heatmap of differentially expressed genes of interest in HCT116 cells. *Genes of interest that were differentially expressed in all Knock Out (KO) cells compared to HCT116^{WT} cells are depicted in the heatmap. Clustering was performed using k-means clustering (k=4). Colour code scheme for standardized gene expression values (z-scores) are indicated in the heatmap colour key.*

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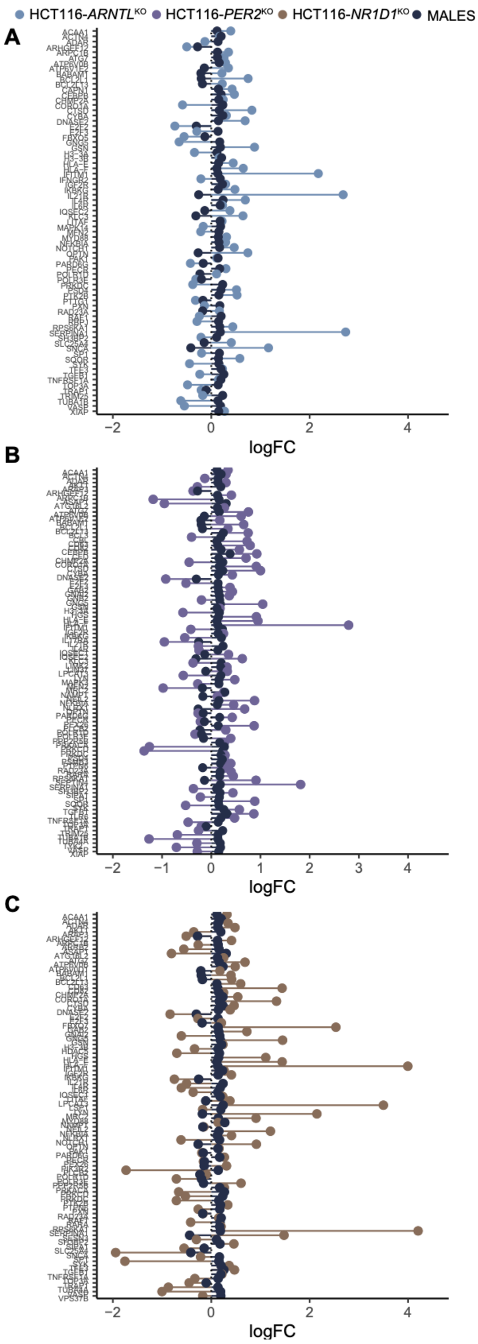

Figure S7. Commonly differentially expressed genes of interest in HCT116 KOs and males in IPD cohort. *Lollipop plots showing the direction of change in mean expression level of genes of interest in* ***(A)*** *HCT116-ARNTL^{KO} cells vs HCT116^{WT} cells and male IPD patients vs controls* ***(B)*** *HCT116-PER2^{KO} cells vs HCT116^{WT} cells and male IPD patients vs controls* ***(C)*** *HCT116-NR1D1^{KO} cells vs HCT116^{WT} cells and males IPD patients vs controls.*

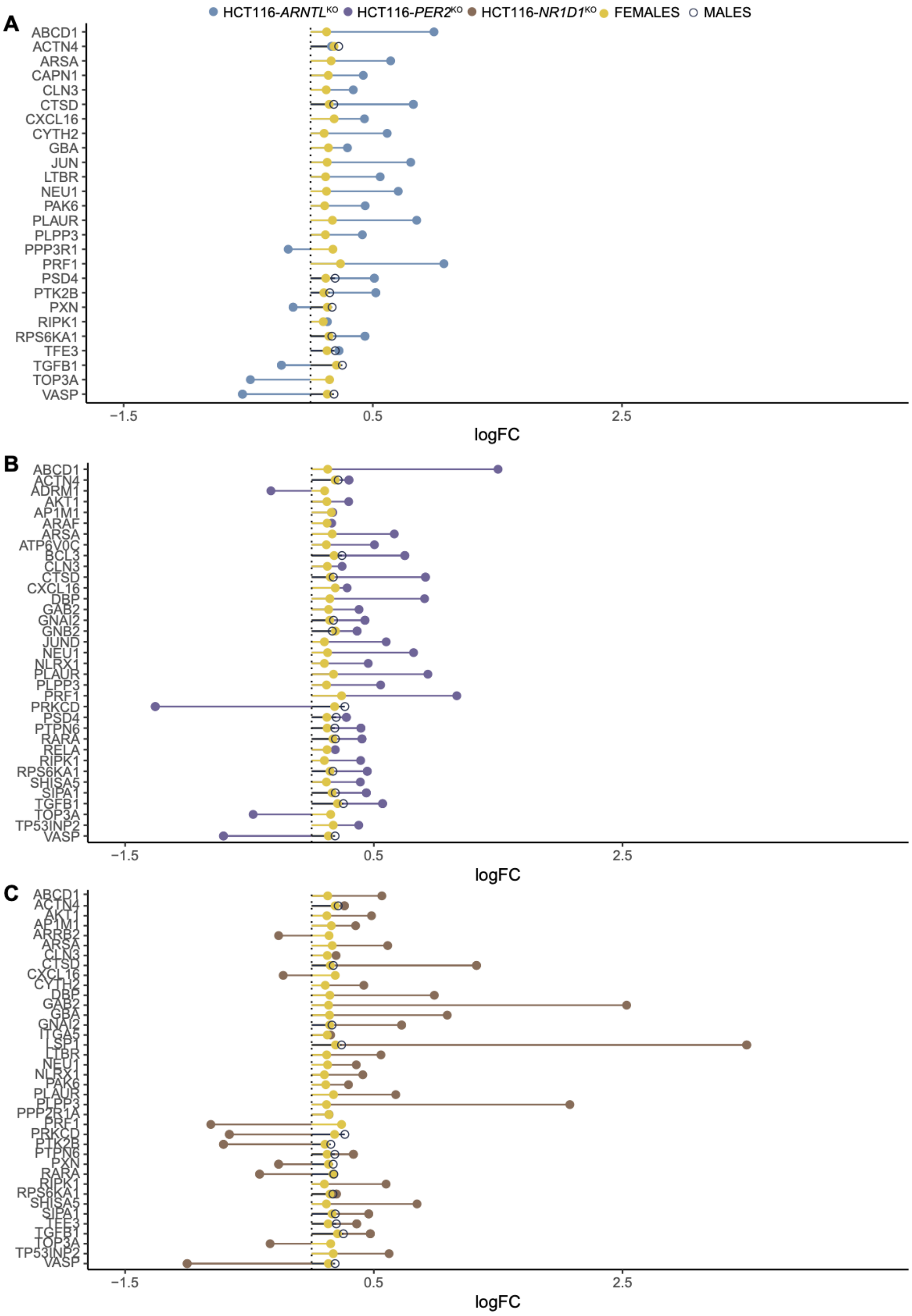

Figure S8. Commonly differentially expressed genes of interest in HCT116 KOs and in females and male cohort. *Lollipop plots showing the direction of change in mean expression level of genes of interest that were found in the intersection between females and males in IPD cohort vs controls and* ***(A)*** *HCT116-ARNTL^{KO} cells vs HCT116^{WT} cells* ***(B)*** *HCT116-PER2^{KO} cells vs HCT116^{WT} cells* ***(C)*** *HCT116-NR1D1^{KO} cells vs HCT116^{WT} cells .*

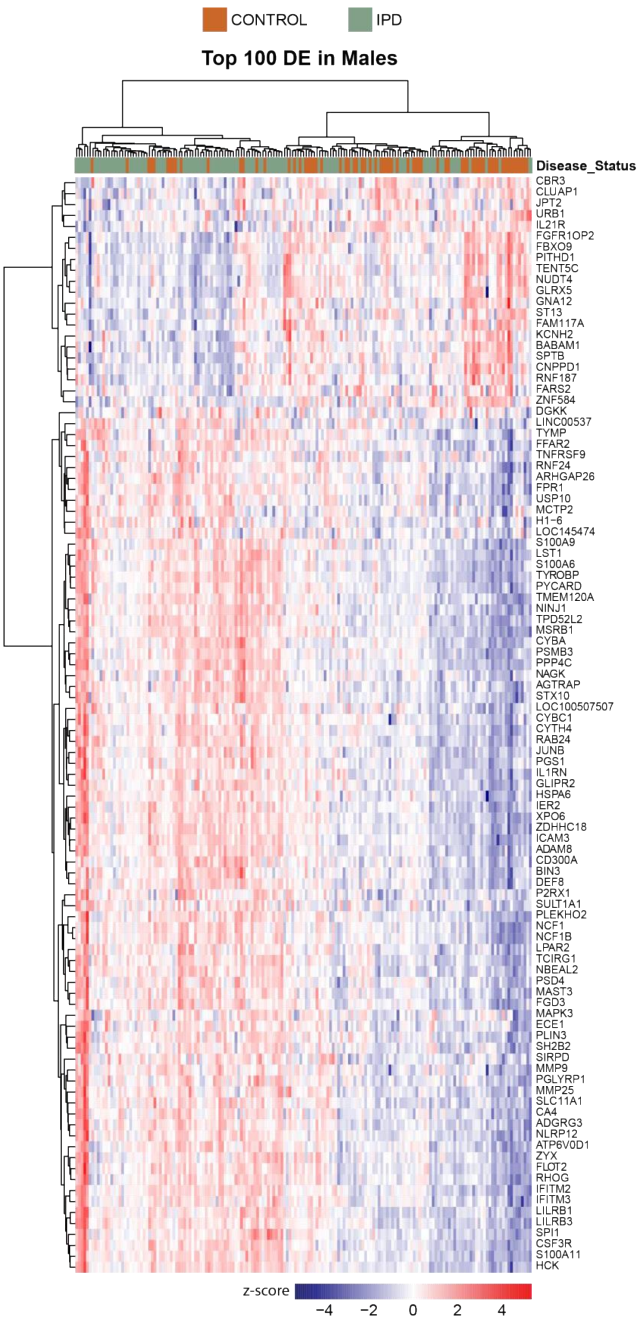

Figure S9. Heatmap of top 100 differentially expressed genes (sorted by p-value) in males. The heatmap was clustered using WardD2 linkage and Euclidean distancing. Disease status of the samples denoted as orange for controls and green for patients. Colour code scheme for standardized gene expression values (z-scores) are indicated in the heatmap colour key.

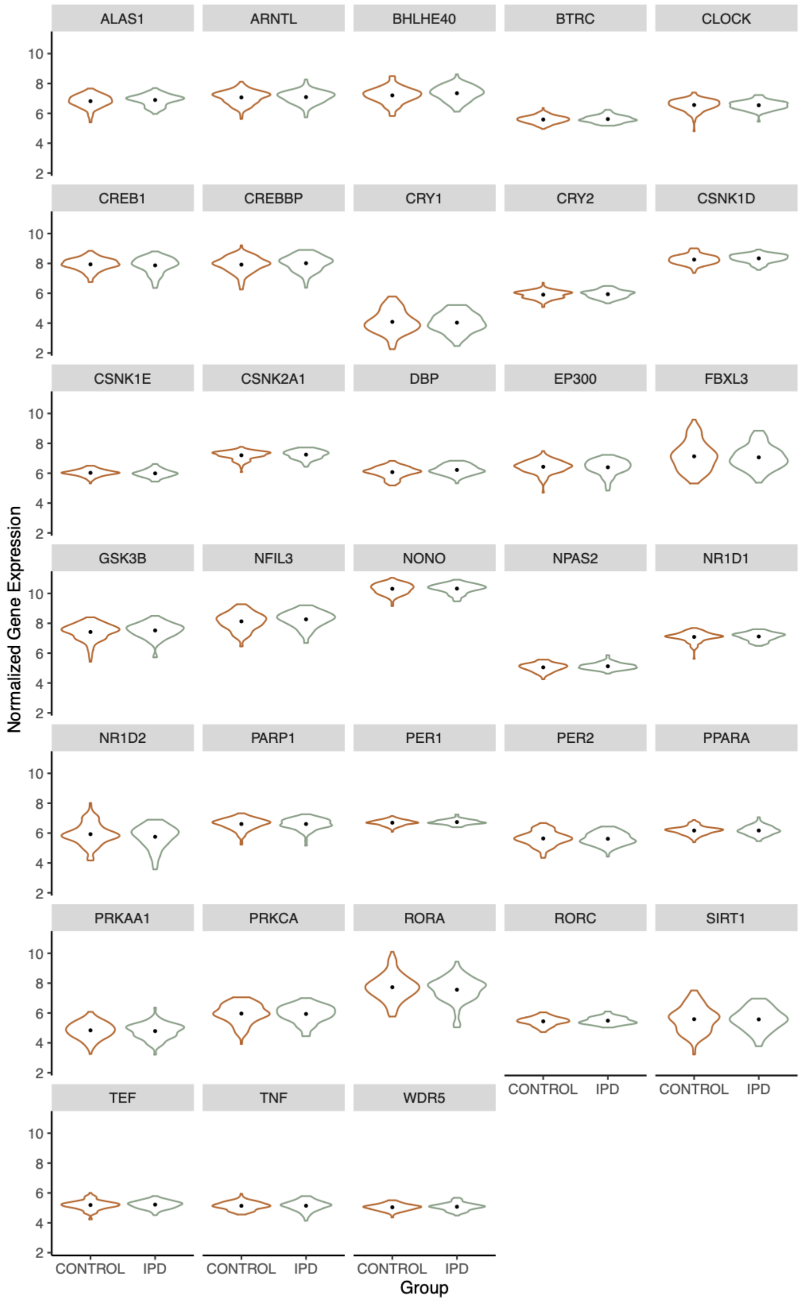

Figure S10. Expression patterns of ECCN genes in female cohort. *Violin plots used to depict the distribution of individual gene expression levels of ECCN genes in females (N_{IPD}=90, $N_{Controls}$=138). Mean gene expression of total population is depicted with black dots. The disease status is denoted as orange for controls and green for patients.*

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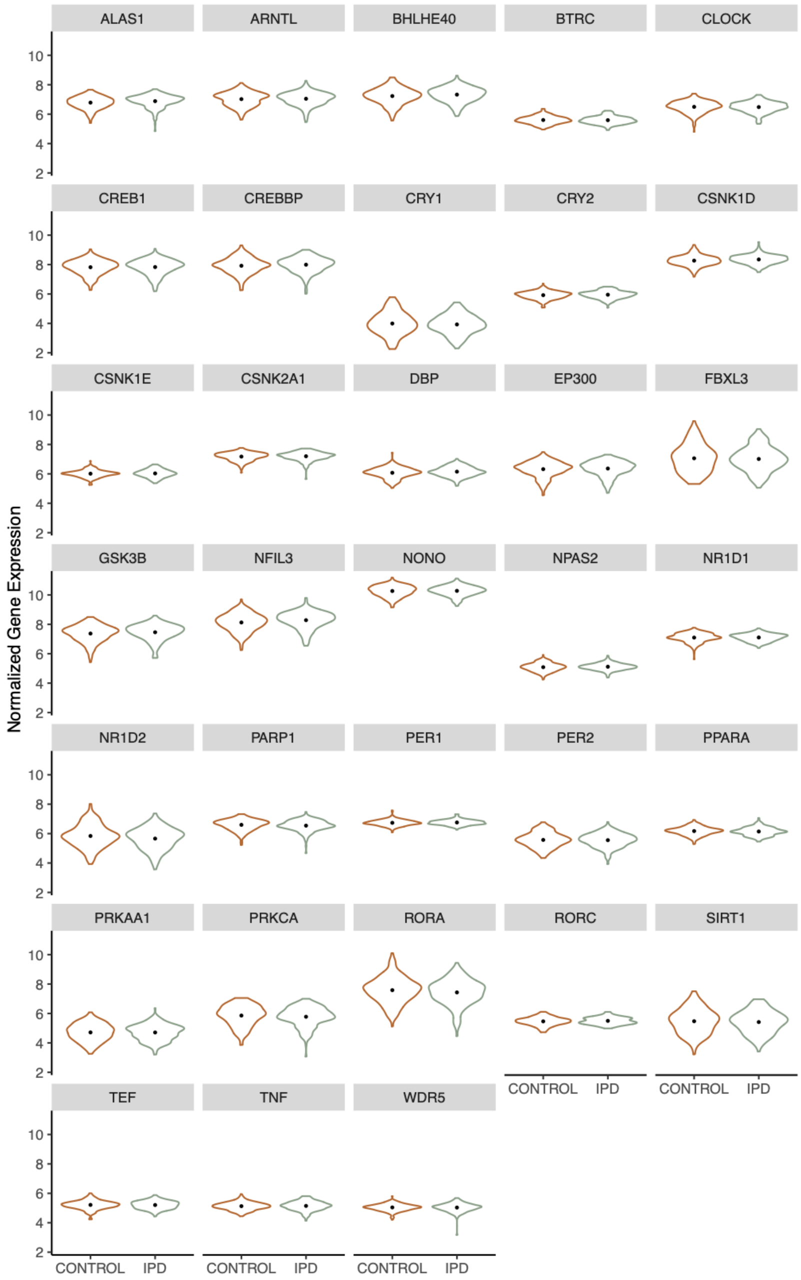

Figure S11. Expression patterns of ECCN genes in IPD cohort. *Violin plots used to depict the distribution of individual gene expression levels of ECCN genes in patient cohort ($N_{Controls}$=227, N_{IPD}=204). Mean gene expression of total population is depicted with black dots. The disease status is denoted as orange for controls and green for patients.*

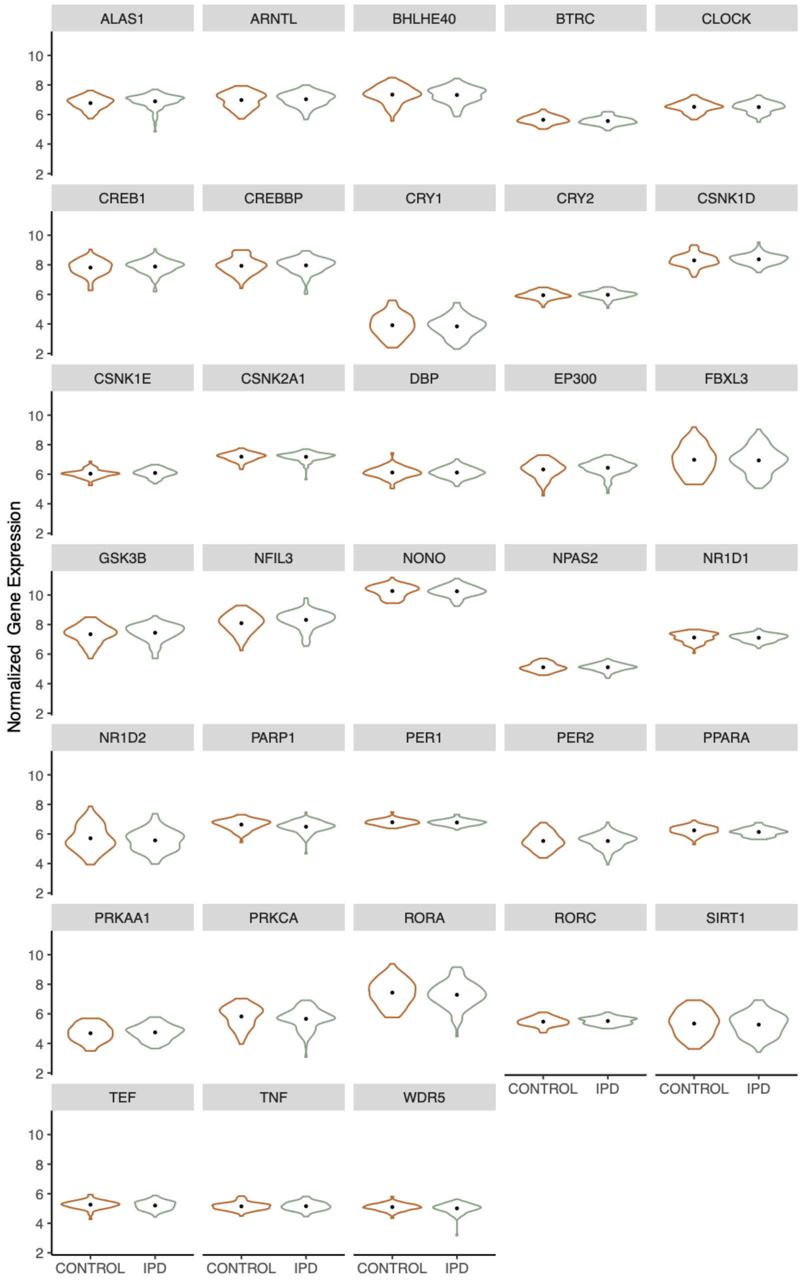

Figure S12. Expression patterns of ECCN genes in male cohort. *Violin plots used to depict the distribution of individual gene expression levels of ECCN genes in males (N_{IPD}=100, $N_{Controls}$=69). Mean gene expression of total population is depicted with black dots. The disease status is denoted as orange for controls and green for patients.*

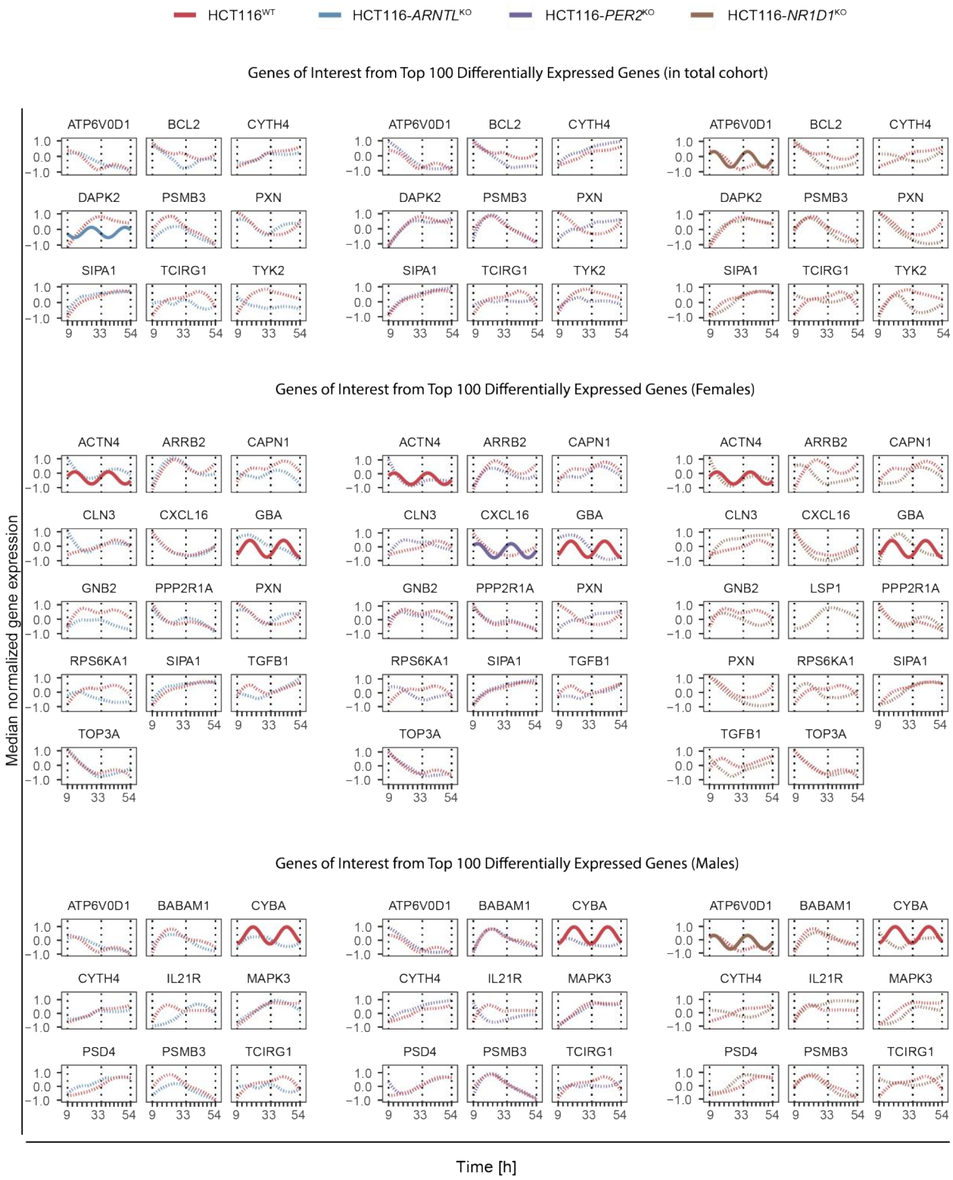

Figure S13. Circadian expression profiles of genes of interest among the top 100 differentially expressed genes in the IPD cohort (without sex separation, top panel), in females (middle panel) and in males (bottom panel). Rhythmicity analysis was computed considering 24h-period. Depicted are significantly rhythmic genes ($q < 0.05$ and relative amplitude □ 0.1) plotted using a harmonic regression fitting (full lines) whereas non-significantly rhythmic genes were represented with LOESS (dashed lines) (see Figure S2 for similar analysis in SW480 and SW620 cells).

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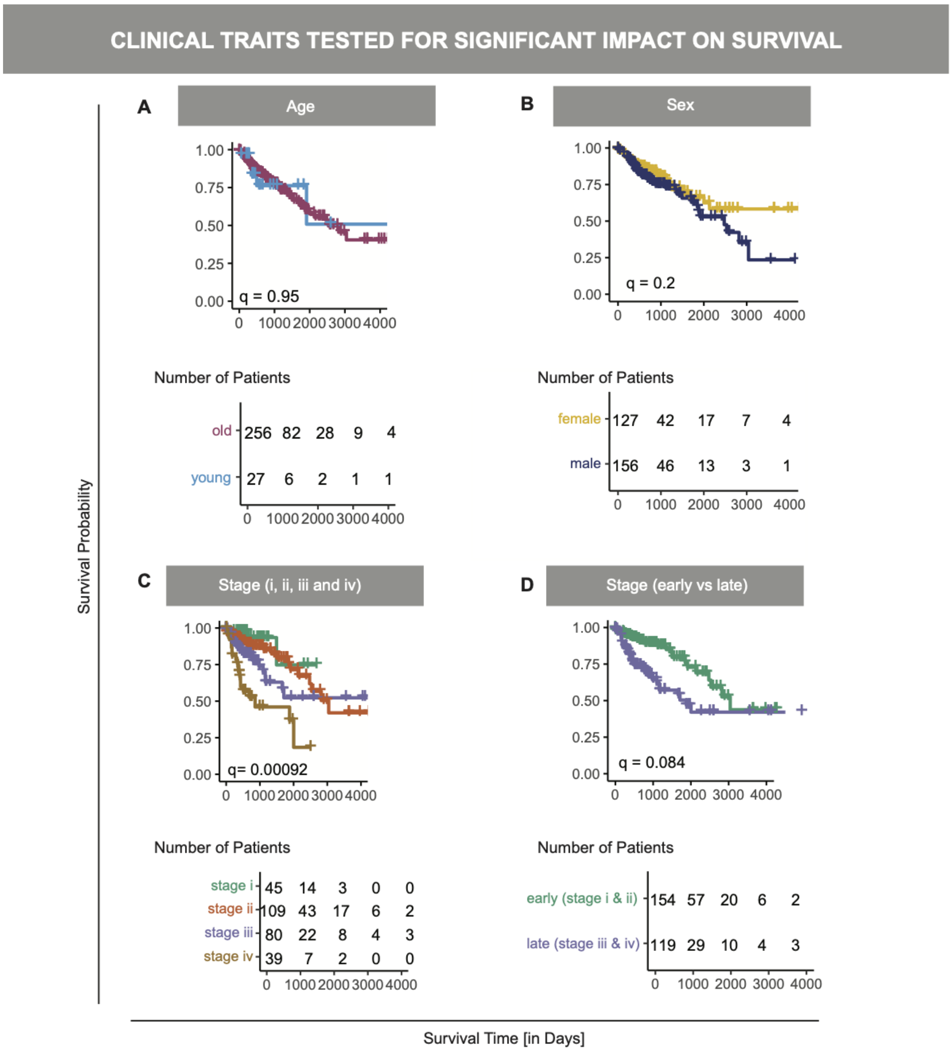

Figure S14. Significance analysis on the impact of clinical traits potentially relevant for patient survival. *Kaplan Meier curves represent patient survival in subgroups of the TCGA COAD cohort based on different clinical trait including **(A)** age ; **(B)** sex ; **(C)** disease stage (I,ii,iii,iv) and **(D)** early stage (I and ii) versus late stage (stage iii and iv).The tables show number of patients (population sizes) in each sub-group for different survival intervals (in days).*

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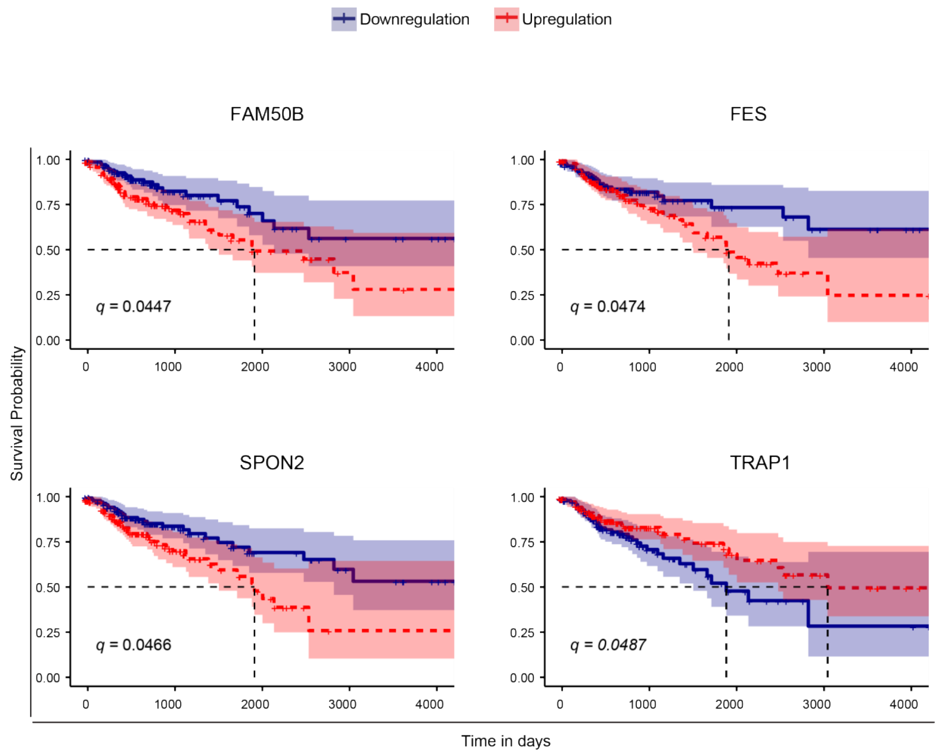

Figure S15. Survival curves of short-listed genes associated with patient survival. Kaplan Meier survival curves for genes found to be common in our analysis and genes associated with patient survival in TCGA COAD cohort. The patient cohort was separated into two groups based on the mean gene expression level as high and low expression group and a subsequent comparison of survival time (in days) was performed. Stage stratiϕed q-values for the survival analysis with cox-regression yielded the results: FAM50B : q =0.312 ; FES : q =0.1 ; SPON2 : q =0.143 ; TRAP1 : q=0.042

Supplementary Table 1. *Demographic information of GSE99039 dataset downloaded from GEO (the date of data retrieval 16.02.2021) and ArrayQualityMetrics outlier test results (failed tests are marked with "x")*

Supplementary Table 2. *The list of genes pre-selected from KEGG pathways in immune system module*

Supplementary Table 3. *The list of genes pre-selected from KEGG pathways DNA replication and repair*

Supplementary Table 4. *The list of genes pre-selected from KEGG pathways involved in cellular growth*

Supplementary Table 5. *The list of genes pre-selected from KEGG pathways involved in redox homeostasis (energy metabolism and catabolism)*

Supplementary Table 6. *The list of Extended Core-Clock Network*

Supplementary Table 7. *The list of genes pre-elected from KEGG pathways involved in Parkinson's Disease and genes involved in PD associated disorders Restless legs syndrome (RLS) with possible circadian influences*

Supplementary Table 8. *GO Biological Process Enrichment results of 24h and 12h rhythmic genes*

Supplementary Table 9. *GO Biological Process Enrichment results of differentially expressed genes in HCT116 cells*

Supplementary Table 10. *GO Biological Process Enrichment results of differentially expressed genes in Parkinson's Disease Dataset*

Supplementary Table 11. *The list of genes resulted from the analysis with CRC and IPD data sets*

Supplementary Table 12. *The list of differentially expressed genes found to be associated with overall survival of TCGA-COAD patients and results from the cox-regression analysis based on stratification for tumor stage*

9.1.2. Printing copy of the publication for Yalçin, M., Mundorf, A., Thiel, F., Amatriain-Fernández, S., Kalthoff, I. S., Beucke, J. C., Budde, H., Garthus-Niegel, S., Peterburs, J., & Relógio, A. (2022). It's About Time: The Circadian Network as Time-Keeper for Cognitive Functioning, Locomotor Activity and Mental Health. Frontiers in Physiology

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It's About Time: The Circadian Network as Time-Keeper for Cognitive Functioning, Locomotor Activity and Mental Health

Müge Yalçin[1,2], Annakarina Mundorf[3], Freya Thiel[3,4], Sandra Amatriain-Fernández[5], Ida Schulze Kalthoff[3], Jan-Carl Beucke[3,6,7], Henning Budde[5]*, Susan Garthus-Niegel[3,4,8]*, Jutta Peterburs[3]* and Angela Relógio[1,2,3]**

[1]Institute for Theoretical Biology (ITB), Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [2]Molecular Cancer Research Center (MKFZ), Medical Department of Hematology, Oncology, and Tumour Immunology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany, [3]Institute for Systems Medicine and Faculty of Human Medicine, MSH Medical School Hamburg, Hamburg, Germany, [4]Institute and Policlinic of Occupational and Social Medicine, Faculty of Medicine, Technische Universität Dresden, Dresden, Germany, [5]Institute for Systems Medicine and Faculty of Human Sciences, MSH Medical School Hamburg, Hamburg, Germany, [6]Department of Psychology, Humboldt-Universität zu Berlin, Berlin, Germany, [7]Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden, [8]Department of Child Health and Development, Norwegian Institute of Public Health, Oslo, Norway

A variety of organisms including mammals have evolved a 24h, self-sustained timekeeping machinery known as the circadian clock (biological clock), which enables to anticipate, respond, and adapt to environmental influences such as the daily light and dark cycles. Proper functioning of the clock plays a pivotal role in the temporal regulation of a wide range of cellular, physiological, and behavioural processes. The disruption of circadian rhythms was found to be associated with the onset and progression of several pathologies including sleep and mental disorders, cancer, and neurodegeneration. Thus, the role of the circadian clock in health and disease, and its clinical applications, have gained increasing attention, but the exact mechanisms underlying temporal regulation require further work and the integration of evidence from different research fields. In this review, we address the current knowledge regarding the functioning of molecular circuits as generators of circadian rhythms and the essential role of circadian synchrony in a healthy organism. In particular, we discuss the role of circadian regulation in the context of behaviour and cognitive functioning, delineating how the loss of this tight interplay is linked to pathological development with a focus on mental disorders and neurodegeneration. We further describe emerging new aspects on the link between the circadian clock and physical exercise-induced cognitive functioning, and its current usage as circadian activator with a positive impact in delaying the progression of certain pathologies including neurodegeneration and brain-related disorders. Finally, we discuss recent epidemiological evidence pointing to an important role of the circadian clock in mental health.

Keywords: circadian clock network, circadian medicine, circadian dysregulation, neurocognitive functioning, neurodegenerative disorders, mental health

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****Correspondence:***
Jan-Carl Beucke
jan.beucke@medicalschool-hamburg.de
Henning Budde
henning.budde@medicalschool-hamburg.de
Susan Garthus-Niegel
susan.garthus-niegel@medicalschool-hamburg.de
Jutta Peterburs
jutta.peterburs@medicalschool-hamburg.de
Angela Relógio
angela.relogio@medicalschool-hamburg.de

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INTRODUCTION

Life on earth has evolved around the approximately 24 h solar day. Accordingly, organisms ranging from plants to mammals, including humans, have developed an endogenous (internal) time keeping machinery–the biological (circadian) clock. The term endogenous here refers to the processes derived from an internal origin of the system itself i.e., from the organism or cell/tissue. Hence, endogenous rhythms refer to rhythms which the organism is able to generate without external input. The clock enables the timing regulation of physiology and behaviour and provides an advantage for adaptation, survival, and anticipation to foreseeable daily changes such as the light and dark cycles (Astaburuaga et al., 2019). These rhythms are circadian (*circa* = about; *dies* = a day) and show a periodicity of approximately 24 h. The timing of cellular processes, regulated by the circadian clock, is crucial for the maintenance of proper organismal homeostasis.

Increased awareness concerning the impact on health caused by perturbations in our internal time keeping machinery has strongly contributed to the growing research in the field. In 2017, discoveries on genetics and molecular mechanisms of the biological clock made over the past decades led to the award of the Nobel Prize for Physiology or Medicine to Jeffrey C. Hall, Michael Rosbash and Michael W. Young (Bargiello et al., 1984; Zehring et al., 1984; Siwicki et al., 1988; Hardin et al., 1990; Liu et al., 1992; Vosshall et al., 1994; Price et al., 1998). Although accumulating evidence points to a crucial role of the biological clock in temporal regulation and optimal functioning of physiological and behavioural processes, clinical applications of this knowledge in the maintenance of health and fighting disease remains scarce. For numerous reasons, ranging from the low number of clinical studies on chronobiology and circadian medicine to the logistic complexity of introducing circadian consideration in the planning of clinical studies and subsequent treatment routines, a limited number of clinical applications has been introduced, one of those rare examples being the use of circadian lighting solutions implemented at intensive care units (Luetz et al., 2019). It is thus timely to better understand the biological clock, as well as the consequences of its failure on human health, and develop ways for applying this knowledge in clinical practice.

Circadian rhythms share certain characteristics, as they are: 1) self-sustained oscillations (~24 h period) present also in the absence of external cues (defined as free running period, FRP); 2) susceptible to resetting by Zeitgebers (timing cues) as defined by pioneers in the field (Pittendrigh and Minis, 1964; Aschoff, 1965); and 3) temperature-compensated. The German term Zeitgeber (literally "time giver") is defined as any external or environmental cue that entrains an organism's biological rhythm to cyclic, repetitive signals to ensure the temporal regulation, adaptation and functioning of the organism. These environmental factors refer to changes in the surrounding environment such as light exposure, temperature, oxygen or nutrient availability, mealtimes, physical exercise, work schedules, and social cues as illustrated in **Figure 1**. Of note, although any environmental factor which can enable a 24 h rhythm may serve as Zeitgeber for other oscillators, light has been denoted as the primary and the most dominant Zeitgeber to the circadian clock (Duffy and Czeisler, 2009; Ashton et al., 2022). The underlying reason being is that light and dark cycles are responsible for all other environmental rhythms therefore serving a reliable source of information to adjust the timely regulation of process across the day (Panda et al., 2002; Roenneberg et al., 2013a). In addition, several internal signals such as daily fluctuations of hormones or metabolites in bloodstream, neurotransmitters or body temperature can also act as 'internal timing cues' in mammals (Dibner et al., 2010).

As mentioned above, circadian rhythms are synchronized to external light signals every day to ensure robust cyclic activity in molecular and cellular processes, which in turn regulate the timing of physiology including core body temperature, sleep/wake cycles, hormonal secretion (e.g., cortisol, melatonin and dopamine), and behaviour (e.g., physical activity and mood) (Koronowski and Sassone-Corsi, 2021) (**Figure 1**). Both genetic (the alterations in of clock or clock-controlled genes) and/or environmental factors (e.g., sleep disruption, artificial light exposure) may disrupt circadian rhythms (Cederroth et al., 2019; Ruben et al., 2019; Walker et al., 2020). Several studies have shown that, in the absence of environmental cues, humans have altered sleep and activity cycles (slightly longer than 24 h), and that genetic variants of certain circadian genes can affect period length (Toh et al., 2001; Xu et al., 2005; He et al., 2009) and phase of behavioural outputs (Archer et al., 2003; Yamaguchi et al., 2015). While homeostatic sleep regulation refers to the trigger to sleep, which is influenced by the duration of wakefulness, the circadian sleep regulation transmits stimulatory signals to circadian networks to promote awakening in opposition to the homeostatic drive to sleep. Thus, homeostatic sleep processes have a strong influence on the maintenance and depth of sleep. According to the well-known two-process model of sleep regulation, which suggests that, this regulation is determined based on the interaction of homeostatic processes (also known as process S), and the prior amount of sleep and fragmentation (by awakening), which is controlled by the circadian clock (process C). Sleep occurs when S approaches its upper boundary, while awakening occurs at its lower boundary (Borbély, 1982; Daan et al., 1984; Borbély et al., 2016).

Environmental cues can influence not only the period, but also the phase and amplitude (peak to trough distance) of circadian oscillations (Roenneberg and Merrow, 2007). The phase of an oscillation is defined as the timing of maximum expression and/or activity within a circadian cycle (24 h). The term "chronotype" on the other hand is used to denominate an individual's subjective, internal timing, and provides a classification of individuals (i.e., morning larks or night owls) based on their preferred sleep-wake phases (Roenneberg et al., 2003; Roenneberg et al., 2019; Hesse et al., 2020). As a result of the different chronotypes, humans show a predisposition to be more efficient at certain activities (e.g., sports, food intake) at different times of day, and following one's chronotype is crucial to maintain optimal functioning of the circadian system.

Continuous alteration of the timing of daily routines, for example due to shift work or travel, can result in the misalignment between internal and external time (**Figure 1**).

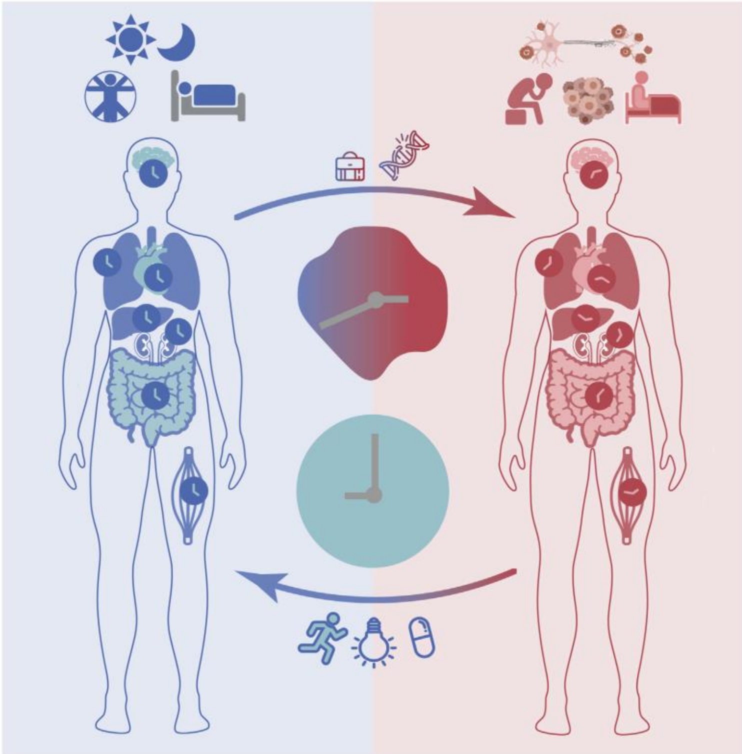

FIGURE 1 | The circadian clock impacts health and disease. In response to environmental cues such as daily light/dark cycles, the master clock in the suprachiasmatic nucleus (SCN) and peripheral clocks are synchronized through complex routes of neuronal and hormonal networks. Proper functioning of the circadian clock ensures correct timing of physiology and behaviour (e.g., sleep/wake cycles) and contributes to maintaining a healthy life. Disruptions of circadian rhythms may occur due to genetic factors or life style determinants such as long-term shift work and are associated with various health complications including mood and sleep disorders, neurodegenerative diseases and cancer. Clinical interventions aiming at restoring circadian rhythms and minimizing the consequences of circadian disruption (e.g., bright light therapy, physical exercise), as well as targeting treatment optimization via timing the administration of drug compounds (i.e., chronotherapy) are currently being evaluated.

The term 'circadian misalignment' in this context can be described as a mismatch between an individual's circadian chronotype and their physical or social environment (e.g., light-dark cycle, school, or work times) (Roenneberg and Merrow, 2016). This means that, if circadian rhythms in all cellular clocks are synchronised to the daily cycle, as well as to each other, they are aligned. On the other hand, if throughout the body, they are termed as misaligned. The resulting disruption of circadian rhythms negatively impacts human health and has been found to be associated with various diseases including sleep disorders (Rijo-Ferreira and Takahashi, 2019), mental disorders (Walker et al., 2020), neurodegenerative diseases (Carter et al., 2021) and cancer (Sulli et al., 2019), as well as metabolic disorders such as obesity (Baron and Reid, 2015) and diabetes (Javeed and Matveyenko, 2018). Although a limited number of interventions are available a few prototypes have been used to prevent and/or reduce circadian disruption in daily activities. For example, special bio-lightening in overseas flights has been implemented to prevent misalignment of the internal clock and the geophysical time while traveling across time zones, a concept described as 'jet lag'. Other examples include exposure to bright light in seasonal depressive disorder, a mental condition related to variation of daily light exposure across different seasons, which has been reported as an effective treatment equivalent to antidepressants (Campbell et al., 2017). Disruptions of circadian rhythms in sleep/wake cycles, blood pressure, and hormonal secretion such as melatonin have been reported in patients with mood disorders. Clinical studies have pointed to a direct association between the severity of circadian rhythm disturbances and mood disorders and further showed a positive impact on mood disorders when restoring circadian dynamics with treatments (Ruben et al., 2019; Sato et al., 2021).

As mentioned above, research in the circadian field has grown considerably over the past years, but usage of this knowledge in the maintenance of health and prevention or treatment of disease remains to be fully explored. Increased awareness of the role of circadian rhythms in health maintenance, as well as non-invasive tools to characterize the individual chronotype, also at the molecular level, are therefore needed.

In this review, we will first provide an overview of the current knowledge regarding the molecular and neuronal circuits that drive circadian rhythms in mammals, with a particular focus on humans, followed by a discussion of recent studies reporting connections between perturbations of the circadian clock and pathological development. We will highlight the link between a dysregulated clock and neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), and describe results from recent studies reporting restoration of circadian rhythms as a means to improve disease-related symptoms (**Section 3.1**). We will further discuss the circadian regulation of behavioural and cognitive functions, memory consolidation, and implication of loss of this regulation in mental disorders such as obsessive-compulsive disorder (OCD), major depressive disorder (MDD), and schizophrenia (**Sections 3.2**). We will elaborate on emerging novel aspects linking the circadian clock to physical exercise and exercise-induced neurocognitive functioning, as well as its current usage as circadian activators with a positive influence on delaying the progression of certain neurodegenerative and brain disorders (**Section 3.3**). Finally, in **Section 3.4**, we will discuss recent epidemiological studies pointing to the important role of the circadian clock in mental health with a particular focus on its link to public mental health and the transition to parenthood.

MAKING BIOLOGICAL TIME: THE MOLECULAR AND NEURAL CIRCUITS MEDIATING GENERATION AND FUNCTIONING OF CIRCADIAN RHYTHMS

The Suprachiasmatic Nucleus as the Master Regulator of the Circadian System

In mammals, the circadian system is hierarchically organized. The central pacemaker of the circadian system is located in the suprachiasmatic nucleus (SCN), a small bilateral structure in the anterior part of the hypothalamus positioned above the optic chiasm. In humans, the core-molecular oscillator is reset with exposure to light in the early morning. Environmental light signals are perceived via intrinsic photosensitive ganglion cells and photic information is transmitted to the SCN via the retinohypothalamic tract. Via complex routes of neuronal and hormonal networks, the SCN entrains peripheral oscillators and modulates the period and phase of all oscillators throughout the body (Bittman, 2021).

The generation of time in the main pacemaker is ensured by clusters of coupled oscillators (~20.000 neurons) spread across two SCN subregions, the core and a shell region surrounding the core, which differ in their expression of specific secreted neurochemicals (Hastings et al., 2018) (**Figure 2A**). The core region governs vasoactive intestinal peptide (VIP)-, calretinin, neurotensin (NT)-, and gastrin-releasing peptide (GRP)-expressing neurons (Slat et al., 2013). VIPs are the most abundant neurotransmitters and are essential for the transmission of light signals. Together with their binding partner VIP-receptor 2 (VIPR2; also known as VPAC2), found in both core and shell regions, VIP neurons regulate the rhythmic expression of other neuronal circuits and ensure the coupling of SCN neurons, as well as non-SCN brain clocks. Perturbation of VIP signalling via knockout (KO) experiments resulted in the disruption of inter-neuronal coupling and rhythmic expression of core-clock genes, and led to the dysregulation of activity and entrainment mechanisms in mouse models, pointing to an essential role of this coupling on physiological processes (Maywood et al., 2011; Edwards et al., 2016).

The shell region, on the other hand, is composed of arginine vasopressin (AVP)-expressing neurons that colocalize with Gamma-Aminobutyric Acid (GABA), a crucial neurotransmitter, which regulates excitability of neurons in the central nervous system (CNS), and plays a role in the phase resetting of other brain (non-SCN) and peripheral clocks throughout the body (Herzog et al., 2017; Mieda, 2019). AVP neurons are under direct regulation of the circadian clock and widely present in the SCN. They can substitute the function of VIP neurons, in the absence of VIP signalling (Maywood et al., 2011). The absence of AVP receptors was shown to weaken the coupling between SCN neurons, and to accelerate re-entrainment of behavioural rhythms in mice (Yamaguchi et al., 2013; Mieda et al., 2015).

In addition, the SCN plays an essential role in the entrainment of peripheral clocks (**Figure 2A**). It has been suggested that a bidirectional interaction may exist between the master pacemaker and other body clocks pointing to the existence of a feedback route from peripheral clocks to the SCN. Although the exact mechanisms underlying this interaction remain to be elucidated, energy homeostasis via the regulation of the hormones ghrelin (involved in short-term appetite regulation) and leptin (considered as a satiety hormone) can modify the activity of SCN neurons (Grosbellet et al., 2015). Also melatonin, a pineal gland rhythmically secreted hormone involved in the regulation of sleep and wake timing, activates its complement receptors (MT_1 and MT_2) in the SCN, thereby regulating the robustness of circadian rhythmic activity (Liu et al., 2016). In turn, melatonin is also under the direct control of the SCN (Cajochen et al., 2003; Zisapel, 2018; Arendt, 2019).

Interestingly, recent studies on *Bmal1* (brain and muscle ARNT-like 1) KO mice showed that besides synchronizing the SCN, light stimuli can synchronize peripheral tissues such as liver (Koronowski et al., 2019) and epidermis (Welz et al., 2019). Although peripheral clocks (e.g., liver and kidney) can maintain endogenous rhythms and be synchronized by other external cues such as feeding, ablation of the SCN in mice lead to a high variation of circadian phases among body clocks (Izumo et al., 2014). Studies on a mouse model with SCN-specific disruption of *Bmal1* have reported intact core-clock oscillations in the liver under light/dark conditions (LD: 12 h light, 12 h dark), but

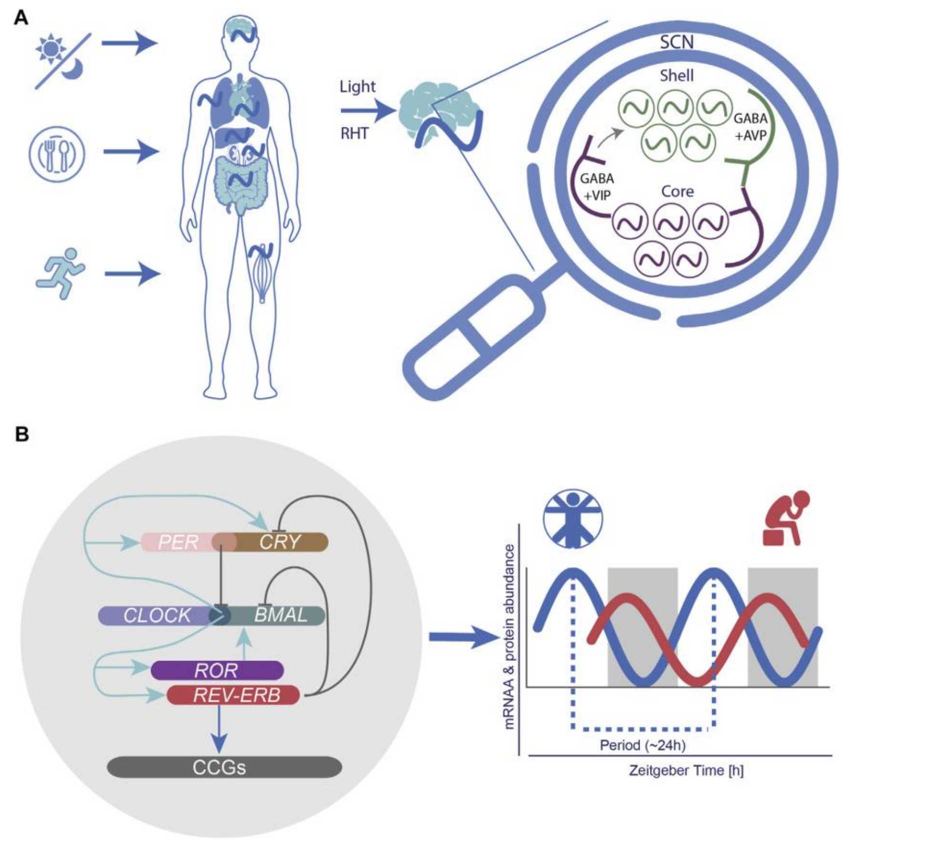

FIGURE 2 | The circadian system as a neural and molecular network and implications for circadian medicine. **(A)** Following light exposure, activated intrinsic photosensitive ganglion cells transmit photic information to the suprachiasmatic nucleus SCN via the retinohypothalamic tract (RHT). The SCN synchronizes peripheral clocks throughout the body via endocrine, neuronal routes and behavioural outputs. The SCN is organized in a "core" (with VIP expressing neurons) and a "shell" region (with AVP expressing neurons), which ensure neuronal coupling. **(B)** At the molecular level the circadian network is formed by intricated self-sustained feedback loops of core-clock elements (CLOCK, BMAL, PERs, CRYs, RORs, and REV-ERBs), which drive 24-h rhythmic oscillations at the mRNA and protein level of several target genes.

dampened after a few days in dark/dark (DD: 24 h dark) conditions (Husse et al., 2014). Altogether, these observations suggest that in the absence of light stimuli, other signals transmitted by peripheral clocks may contribute to the maintenance of 24 h rhythms.

The Molecular Clock Network and Clock-Controlled Cellular Processes

At the cellular level, the mammalian SCN and other peripheral clocks are molecular oscillators consisting of self-sustained transcriptional and translational feedback loops (TTFLs). In humans, the TTFL is composed of genes and proteins, which form positive and negative feedback loops. At the beginning of each circadian cycle, circadian locomotor output cycles protein Kaput (*CLOCK*) heterodimerizes with brain and muscle ARNT-like 1 (*ARNTL*, also known as *BMAL1*) and the complex binds to the enhancer regulator sequences (E-boxes) in the promoter regions of their target genes period (*PER*s), cryptochrome (*CRY*s), nuclear receptor subfamily 1 group D member 1 (*REV-ERBα*, also known as *NR1D1*) and nuclear receptor subfamily 1 group D member 2 (*REV-ERBβ*, also known as *NR1D2*), and retinoid-related orphan receptors (*RORs)* to activate their expression. Additional interconnected feedback loops consist of positive (*ROR*s) and negative (*REV-ERBs*) elements and ensure the robustness and precision of the circadian oscillator (i.e., amplitude and phases) by fine-tuning the transcriptional activity of *BMAL1*. *ROR*s and *REV-ERB*s regulate the activation and repression of *BMAL1* respectively by acting on the REV response element (RRE) sequences.

In humans, at dawn, following the accumulation of *PER* and *CRY*, the PER/CRY heterodimer complex forms and suppresses BMAL1/CLOCK-mediated transcription in the nucleus (**Figure 2B**). Once the PER/CRY complexes are degraded, a new circadian cycle (~24 h) is initiated (Partch et al., 2014). The molecular clock is also influenced by posttranslational modifications such as phosphorylation of PER via the casein kinase 1 (CK1) gene family in order to adjust proper timing of its activity (Etchegaray et al., 2009; Lee et al., 2011). Altogether, these interconnected elements drive and ensure the robustness of 24 h rhythmic oscillations in the expression of various additional target genes also known as clock-controlled genes (CCGs) (**Figure 2B**). CCGs are involved in various cellular mechanisms, including metabolism (Eckel-Mahan and Sassone-Corsi, 2013; Fuhr et al., 2018; Reinke and Asher, 2019; Sato et al., 2022), RNA processing (El-Athman et al., 2018; Yeung et al., 2018; El-Athman et al., 2019), cell cycle (Shostak et al., 2016; El-Athman et al., 2017; Farshadi et al., 2020), DNA damage response (Ando et al., 2001; Sancar et al., 2010; Preußner et al., 2017), and apoptosis (Lee and Sancar, 2011), which are often dysregulated in the context of pathological development. The circadian clock has also been reported to directly modulate the cell cycle via regulation of checkpoints such as *Myc* (Altman et al., 2015; Shostak et al., 2016), *Wee1* (Matsuo et al., 2003), *p21* (Gréchez-Cassiau et al., 2008), and *p16* (Kowalska et al., 2013; El-Athman et al., 2017).

Alterations in core-clock elements have been reported to interfere with the clock network and impact its functioning. In mice, *Bmal1* KO led to the disruption of proper circadian clock functioning and resulted in a shorter lifespan accompanied by an early aging phenotype (Kondratov et al., 2006). Rhythmic behavioural activity in mice was abolished by the simultaneous KO of *Per1* and *Per2* (Zheng et al., 2001) and *Cry1* and *Cry2* (Horst et al., 1999). Mice lacking *Rev-Erbα* continue to exhibit rhythmic behavioural output when measured via wheel running activity, but with a shorter period compared to wild type mice (Preitner et al., 2002), whereas the simultaneous loss of *Rev-erbα* and *Rev-erbβ* led to loss of rhythmicity (Bugge et al., 2012; Cho et al., 2012), evident by a drastic change in the wheel-running activity. Altogether, these results highlight the essential role of the circadian system in the regulation of cellular processes, physiology, and behaviour in mammals.

The Disruption of Circadian Clock Elements and its Impact on Disease

Both, genetic (e.g., aberrant activity of clock or clock-controlled genes) and/or environmental factors (e.g., sleep disruption, artificial lighting) may result in the disruption of circadian rhythms and impact human health (Rijo-Ferreira and Takahashi, 2019). Moreover, the above-described circadian regulated cellular processes, including DNA repair mechanism, metabolism, and cell proliferation are frequently altered in several diseases including sleep disorders, neurodegenerative diseases (AD and PD) (Hood and Amir, 2017b; Carter et al., 2021), cardiovascular diseases (Crnko et al., 2019), obesity (Noh, 2018), diabetes (Javeed and Matveyenko, 2018), autoimmune disorders (Inokawa et al., 2020), and cancer (Hood and Amir, 2017b; Battaglin et al., 2021).

The involvement of the circadian system in the regulation of cellular pathways, which are frequently altered in neurodegenerative diseases and cancer, has been proposed using cancer cellular models (Maiese, 2017; Pacelli et al., 2019). Recently, a time course analysis using human colorectal cancer (CRC) cells with distinct clock phenotypes pointed to a link between cancer and neurodegenerative diseases involving the circadian system, and suggested the differential enrichment of genes involved in HD, AD and PD (Yalcin et al., 2020). In a follow-up study, the KO of core-clock genes in CRC cell lines was shown to disrupt rhythmic expression of cancer and neurodegeneration-related genes and led to similar alterations at the mean gene expression level, as observed in a cohort of PD patients compared to healthy controls (Yalcin et al., 2021). Circadian disturbances are among the earliest symptoms of neurodegenerative diseases such as HD, AD, and PD, and malfunctioning in the molecular mechanisms of the circadian system is thought to play a pivotal, and possibly even causal, role in their pathological development (Hood and Amir, 2017b; Carter et al., 2021). Interestingly, the usage of Zeitgebers (e.g., light) seems to support the treatment of several neurodegenerative disorders (Maiese, 2017; Carter et al., 2021).

Neurodegeneration is typically characterized by the loss of proper neuronal functioning due to excessive neuronal cell death and structural aberrations in protein configuration, such as amyloid formation as a result of accumulated alpha-synuclein in genetic forms of PD. Consequently, patients with neurodegenerative diseases show a variety of symptoms including deficits in motor control and cognition, mood disorders, or sleep dependent symptoms. A well-known example pointing to the dysregulation of the circadian system in neurodegenerative diseases is the worsening of symptoms such as increased cognitive malfunctioning, disruption of sleep profiles, failure in thermal regulation, and mental breakdowns leading to psychotic symptoms such as confusion and hallucinations in some patients during early evening hours, also known as 'Sundown Syndrome' (Bliwise et al., 1995; Volicer et al., 2001; Canevelli et al., 2016).

Genetic polymorphisms in clock genes have been linked to certain disease phenotypes, in particular in PD. A study by Gu and colleagues in which samples from 1394 PD patients and 1342 controls were genotyped reported that *ARNTL* (rs900147 variant) polymorphism was associated with tremor-dominant phenotype, and *PER1* (rs2253820 variant) polymorphism was linked to postural instability (Gu et al., 2015). In another large cohort study with 646 PD patients, *CLOCK* (3111T/C variant) was found to be associated with sleep disorders and deficits in motor functioning (Lou et al., 2018). In addition, the loss of circadian rhythmicity in *ARNTL* can play a role in Aβ production and plaque accumulation, an event observed in AD progression (Ma et al., 2016). In samples derived from brain tissues of post-mortem AD patients, alteration in rhythmic *ARNTL* methylation activity was reported, suggesting that perturbations in *ARNTL* functioning may contribute to behavioural and cognitive deficits in affected individuals (Cronin et al., 2017). Other studies have also pointed to the existence of a bidirectional interaction between Aβ and the clock machinery in which Aβ can cause alteration of *ARNTL* degradation, thereby disrupting clock functioning and suggesting a complex interaction between AD and the dysregulation of the circadian system (Song et al., 2015; Kress et al., 2018).

The use of interventions to revert perturbations of the circadian system was reported to ameliorate symptoms and diminish the progression of various neurodegenerative diseases. For example, exposure to bright light has been used to reverse the effect of circadian disruption in individuals with PD and AD, as well as in patients with dementia (Hood and Amir, 2017b). In a pilot study, 12 PD patients exhibiting, non-motor symptoms (insomnia and/or depression) in addition to motor PD symptoms received bright light therapy (BLT) (Willis and Turner, 2007). Patients were exposed to BLT at 1,000–1,500 Lux for 60–90 min before their usual sleep time (around 22 h for most patients) over a two-to five-week period. The results suggested an improvement of non-motor symptoms such as sleep onset and fragmentation, as well as an anti-depressant like effect improving their mood. In some patients, additional benefits were observed regarding primary PD symptoms such as slowness of movement (e.g., bradykinesia) and structural rigidity. The enhancement in psychological effects and motor symptoms was confirmed in a longitudinal follow up study, in which a larger number of patients was recruited (120 PD patients; BLT for 1 h between 20 and 22 h) (Willis et al., 2012). Motor symptoms were assessed based on three different coordination tasks and performed during daytime. Yet, an improvement was observed only in patients undergoing the light exposure protocol, and in case of withdrawal of the treatment, no improvement was observed in the test results. Another clinical study investigated the effect of BLT in a PD cohort consisting of 31 patients -receiving dopaminergic treatment (DT). Over a 2-week period, participants received 1 h BLT twice a day (morning and afternoon light exposure scheduled between 9-11h and 17–19 h) or dim-red light therapy as a control condition. Sleep quality was assessed via self-report questionnaires (Epworth Sleepiness Scale score; Pittsburgh Sleep Quality Index; the PD Sleep Scale score) (Videnovic et al., 2017). Results showed improved sleep quality metrics such as sleep fragmentation and daytime sleepiness for patients subjected to BLT. Although results from the above-mentioned studies point to a positive effect in PD symptoms resulting from circadian restoration, a characterization of the circadian clock at the gene expression level is less explored.

In a recent clinical study assessing potential alterations in circadian gene expression, hair follicles were collected from a cohort of PD patients (N = 16). The study evaluated the expression of three core-clock genes *NR1D1*, *NR1D2,* and *PER3* in comparison to a control group. Patients received BLT once a day in the evening (19–21 h) for approximately 3 months and hair samples were collected prior to and following the BLT protocol with a sampling interval of 6 h during one circadian day (Endo et al., 2020). A significant correlation between the peak expression time of *PER3* before and after BLT was documented, which revealed a significant phase delay in most patients following the BLT treatment (Endo et al., 2020). In addition, while no significant association was found between sleep related parameters using the Epworth Sleepiness Scale (ESS) (Johns, 1991), a self-report measure to assess daytime sleepiness, a correlation of sleep improvement with the delayed phase of *PER3* was reported in PD Sleep Scale 2 scores (PDSS-2), a metric to quantify the level of sleep disturbance (Endo et al., 2020).

One potential explanation concerning the role of circadian disruption in neurodegenerative diseases could relate to the change in circadian rhythms phenotype across the life span, which is mirrored in changes in individual chronotypes. Although circadian rhythms are established during early age, changes occur throughout the lifespan (Logan and Colleen, 2019). While the circadian clock of the fetus is synchronized to maternal rhythms until birth, following birth this synchrony is lost and only later re-established (within 3–5 months), once the SCN has synchronized to external light stimuli (Hood and Amir, 2017a; Logan and Colleen, 2019). Children have an early chronotype, but following puberty onset, the circadian rhythms shift. Consequently, "eveningness" tends to peak during late teens or young adulthood. With age, circadian rhythms shift back to an earlier phase, ultimately leading to the "morningness" phenotype commonly observed in healthy adults.

Aging is associated with detrimental changes in the circadian time keeping machinery (Panagiotou et al., 2021), and linked to the pathologies mentioned above. Elucidating the mechanisms underlying aging-induced circadian dysfunction is thus extremely important. In the elderly, functioning and robustness of circadian rhythms is altered, which can be partially explained by reduced sensitivity to light stimuli (Hood and Amir, 2017a), leading to a misalignment of circadian rhythms to environmental day/night cycles and subsequently to circadian disruptions in physiological and cellular processes such as poor sleep, mistiming of biochemical events such as hormonal release (e.g., melatonin), or antioxidant production which is commonly observed in the elderly, as well as in patients with neurodegenerative disorders (Musiek et al., 2013; Singh et al., 2017; Wang et al., 2018; Carter et al., 2021). Moreover, clinical studies describe 24 h rhythmic fluctuation of symptoms, particularly as PD progresses: some patients report more troublesome motor symptoms in the evening (Parkes et al., 1981; Lees, 1989); decreased response to evening doses of levodopa, a commonly prescribed anti-parkinsonian drug (Bonuccelli et al., 2000); around 70% complain of night time akinesia (Bhidayasiri and Trenkwalder, 2018); and some describe morning improvement in motor or non-motor symptoms, the so called "sleep-benefit" (van Gilst et al., 2013). It is therefore essential to characterize the individual's internal circadian rhythm and to adjust external/internal factors in order to avoid or overcome circadian rhythm disorders. In addition, the time for certain activities (taking medication) can be optimized based on the internal timing.

In a recent bioinformatics study pointing to molecular clock dysfunction in PD (Yalçin et al., 2021), PD patients exhibited weaker correlations in the expression of clock genes than in age and sex matched controls. In a normally functioning cell or organism, expression of clock genes is tightly ruled by intricated feedback loops and entrained by the master clock in the SCN. The reduced correlation seen in PD thus supports a dysfunction in these regulatory mechanisms. To further illustrate the potential impact of circadian disruption on neurodegeneration-related genes, we analysed the expression profile of the dopaminergic receptor DRD1 (Dopamine Receptor D1) and Huntingtin (HTT) upon perturbation of core clock genes using a recently published transcriptomics time series RNAseq data (ArrayExpress: E-MTAB-9701; (Yalçin et al., 2020)) obtained from HCT116 wild type and core-clock KO human colorectal cancer cells. *DRD1* showed a differential rhythmic expression upon perturbation of the core-clock (**Figure 3A**). DRD1 is tightly linked to PD, as aberrant interactions between different dopamine receptors including DRD1 in the brain were shown to be involved in L-dopa-induced dyskinesia (Lanza et al., 2021) and risk of hallucination, and circadian factors might also be involved in this interplay. Notably, HTT, whose mutation leads to HD (Li et al., 2003), also exhibited differential rhythmicity (**Figure 3A**). These two examples show that alterations of specific core-clock genes can indeed lead to the disruption of the normal circadian profile in relevant neurodegeneration-related genes.

The therapeutic potential of the circadian clock, also referred to as chronomedicine, gained increasing attention in recent years. Chronomedicine aims at using alternative routes for prevention, drug development, diagnostics, and treatment with a particular focus on the biological clock. A broad range of therapies and their link to circadian rhythms have been investigated mostly in animal models, including therapies against allergies, arthritis, asthma, hyperlipidemia, hypertension, cancer, and neurodegeneration. Yet, clinical studies using and aiming at validating such knowledge remain insufficient (Zhang et al., 2014). Measuring the expression of core-clock and/or clock-controlled genes involved in drug metabolism is thus extremely relevant and can be used to identify novel drug targets and to time drug administration.

To illustrate the discrepancy between basic circadian medicine-related research and published clinical studies involving circadian considerations, we carried out a PubMed search and retrieved all publications between 1 Jan 2011 and 31 Dec 2021 using the search terms: “circadian clock”; “chronobiology""; “biological clock”; “circadian rhythm”; “chronomedicine”; “chronotherapy”; “circadian medicine”;

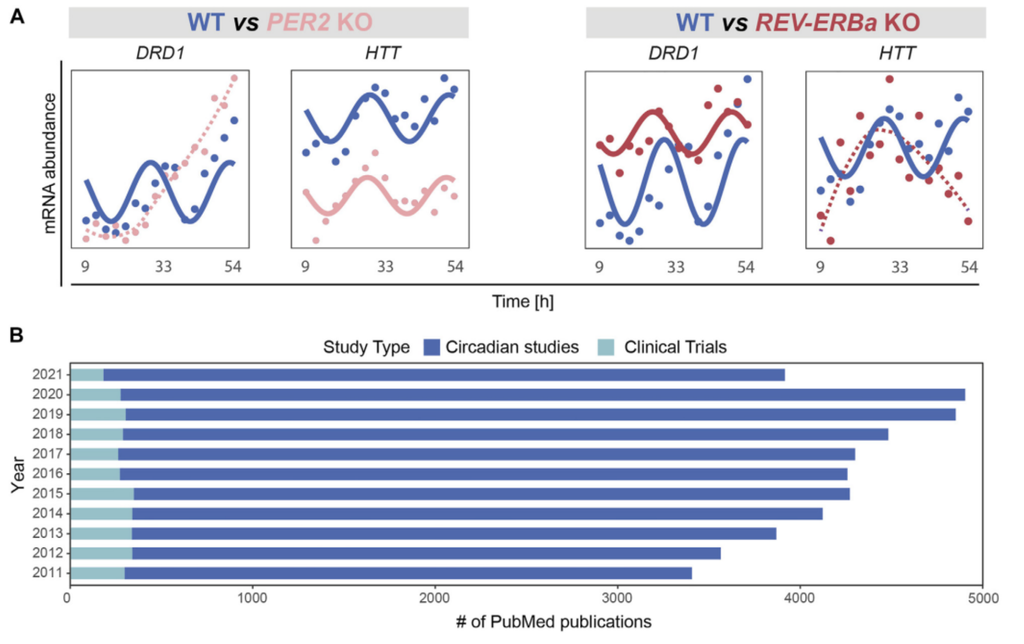

FIGURE 3 | Emerging role of the circadian clock in regulation of disease associated mechanisms and its applications in basic vs. clinical research. **(A)** Perturbation of core-clock genes in an *in vitro* CRC model (HCT116 wild type (WT) and their derived *PER2* and *REV-ERBα* knockout (KO) cells, ArrayExpress: E-MTAB-9701 (Yalcin et al., 2021)) may result in complete abolishment of circadian rhythmicity and/or alteration of oscillatory properties (amplitudes or phases), as observed for *DRD1*, and *HTT*. **(B)** Number of PubMed publications in last 10 years. Studies were categorized based on research type: circadian (studies with basic research) and clinical (studies including circadian biology in a clinical study setup).

"chronotherapies". We selected publications in English, with a full-text link available for further analysis. We first focused only on original research articles including all PubMed references published in circadian research based on human studies (including *in vitro* evidence) and excluded review papers, as well as clinical studies. Subsequently, by including only clinical studies, we identified the number of publications in which "circadian medicine" had been used as an intervention. Our analysis is in line with a previous report by Ruben and colleagues (Ruben et al., 2019) and highlights the fact that the lack of circadian considerations in study protocols remains, while it is urgently needed to allow for circadian medicine to be integrate into clinical practice (**Figure 3B**). For the future implementation of chronotherapy, the integration of genomics and physiological data will be necessary to understand healthy and pathological dynamics at a temporal level. In the following (**Sections 3.2**), we will focus on the circadian regulation of cognitive functioning in health and provide a summary of the latest evidence from mammals and how the loss of this tight regulation is implicated in mental disorders.

CIRCADIAN REGULATION OF BEHAVIOUR AND COGNITIVE FUNCTIONING

As mentioned above, biological (circadian) clocks regulate the timing of many different physiological processes including immune responses, feeding, sleep/wake cycle, reproduction, and the release of hormones such as glucocorticoids. In addition, the circadian clock is also involved in mental processes such as memory consolidation, mood, and reward (McClung, 2013; Logan and Colleen, 2019). In **Section 3.1**, different genetic and environmental factors influencing the circadian timekeeping system such as daylight, food intake, and social interaction are discussed. These influences might then alter certain circadian rhythms, which in turn are associated with dysfunction of cognitive processes. In this section, different behaviours and cognitive processes will be discussed in the context of circadian regulation and circadian implications associated with mental disorders.

A review of existing literature showed that in evening types, aggression and antisocial behaviour were increased compared to morning persons (Schlarb et al., 2014). Similar results were found in adults (Hood and Amir, 2018). A study involving 1,000 adult participants documented that adults identifying as evening types showed higher levels of self-reported impulsivity and anger than morning types (Hwang et al., 2016). Similarly, a study including 641 adult participants reported an association between evening type and higher levels of depressive, irritable, anxious, and cyclothymic (instable mood) behaviour, while morningness was associated with a hyperthymic (e.g., increased energy) temperament (Park et al., 2015). In a web-based study, in which participants were recruited and participated anonymously and online without any interaction with the researchers, multiple questionnaires were used to assess chronotype and temperament (Chrobak et al., 2017). A total of 618 subjects completed the Composite Scale of Morningness (CSM) (Horne and Östberg, 1976), the 72-item Sleep Wake Pattern Assessment Questionnaire (SWPAQ) (Putilov et al., 1990; Putilov and Putilov, 2006) for chronotype and the temperament evaluation of Memphis, Pisa and San Diego-Autoquestionnaire (TEMPS-A) (Akiskal et al., 2005). Interestingly, only an association between hyperthymic temperament and morningness was found, indicating that people with an earlier wake up time showed higher vigilance and wakeability (Chrobak et al., 2017).

Evidence for circadian fluctuations in aggressive behaviour has also been found in clinical populations. For instance, aggressive behaviour reported in some patients suffering from neurodegenerative disorders, such as AD and PD, shows a daily pattern: aggressive verbal or physical outburst and agitated motor behaviours were found to be increased in late afternoon and early evening (Bachman and Rabins, 2006).

However, it has to be noted that observational studies with human subjects, either in clinical or healthy samples, only provide correlational insights, whereas causal relationships between temperament or aggression and chronotype or seasonal period cannot be established. Nevertheless, the stable association reported by studies with sufficiently big sample sizes is intriguing and warrants further systematic investigation.

Interactions of Circadian Rhythms and Food-Related Behaviours

Extensive research has been dedicated to elucidating interactions of circadian rhythms and food-related behaviour. Of note, in this line of research, food intake can be either the dependent variable, e.g., the effect of sleep deprivation on food consumption may be assessed, or an influencing factor. Studies on the latter have used different approaches to investigate the impact of food intake on sleep/wake patterns, activity levels, and more, e.g., by restricting the time periods in which food is available, or by assessing how the timing of food intake affects activity.

Scheer and colleagues investigated whether hunger exhibits a circadian rhythm in healthy participants (Scheer et al., 2013). They monitored 12 participants (6 male and 6 female) over 13 days regarding hunger and appetite ratings while controlling for sleep periods, and most of the potentially influencing environmental factors. Specifically, they controlled for meals (eucaloric, and subjects were required to consume all their food), sleep, activity, posture, room temperature, and light (by subjecting participants to a 'forced desynchrony' protocol). Subjects were also asked to rate their hunger, appetite, and food preferences via computerized visual analogue scales at 5 fixed times within each of the wake periods. The study revealed that hunger followed an endogenous circadian rhythm with less hunger in the morning and the greatest hunger in the evening. Similar rhythms were also revealed for appetite (Scheer et al., 2013).

Given the endogenous rhythm of hunger, the question arises whether insufficient sleep might affect hunger or eating behaviour and thereby, desynchronize this rhythm. Along these lines, Brondel and colleagues analysed whether sleep deprivation affects food consumption and physical activity (Brondel et al., 2010). A total of 12 healthy young adult men were subjected to one night of sleep deprivation and an 8 h sleep period in two different sessions and under two different conditions (sleeping at home or in the laboratory). On the following day, standardized meals were served and physical activity was measured throughout the day. After one-night of sleep deprivation, subjects consumed 22% more energy (kcal), reported greater levels of hunger before breakfast and dinner, and showed more physical activity (Brondel et al., 2010). Pleasantness of food or desire to eat were not affected by sleep deprivation (Brondel et al., 2010). Similarly, Markwald and colleagues investigated the effects of 5 days of insufficient sleep on energy intake in 16 adults (Markwald et al., 2013). Insufficient sleep increased energy needs, physical exhaustion, and food intake above the needed energy levels, and led to weight gain in women as it reduced dietary restraints (Markwald et al., 2013). Furthermore, in this study, participants ate less for breakfast but more throughout the day and after dinner. Thus, the results underline that insufficient sleep is a risk factor for weight gain and obesity. Given the increasing prevalence of insufficient sleep and obesity in modern society, researchers seek to further disentangle the impact of chronic insufficient sleep on the circadian rhythm of subjective hunger and physiological metabolites. McHill and colleagues assigned 15 young adult participants to either control (1:2 sleep:wake ratio) or chronic sleep restriction (1:3.3 sleep:wake ratio) in a 20h/day design for 6 consecutive days over 32 days (McHill et al., 2018). Subjective ratings of hunger were assessed before and after eating, and fasting hormone concentrations were measured after awakening (McHill et al., 2018). Interestingly, hunger and hormone concentration showed a circadian rhythm but did not differ between the two conditions, indicating a limited effect on the rhythmicity (McHill et al., 2018).

It must be noted that all studies mentioned above had small samples sizes and thus, the reported results should be interpreted with caution. Nevertheless, their findings underscore a circadian rhythmicity in hunger and food intake in humans, with sleep deprivation causing potential alterations in this rhythm. The influence of the sleep/wake rhythm on food intake has also been studied extensively in rats and mice (Escobar et al., 2011; DePoy et al., 2017). Studies in wildtype mice have revealed that altered food intake during normally inactive phases (in rodents during the day) shifts the body's circadian rhythm towards meal time, leading to asynchrony between internal and external (light/dark) cycles (Escobar et al., 2011).

Generally, some organs are more sensitive to food-induced signals than others. In rats, for example, the pineal gland reacts to the LD cycle as well as to feeding schedules (Feillet et al., 2008; Wu et al., 2008) whereas the liver is only activated by feeding schedules, and the heart and the adrenal gland, in turn, respond to signals from the SCN (Kornmann et al., 2007) (see **Figure 2**). Timing of food intake is thus a relevant zeitgeber, and impacts the regulating gene expression (Escobar et al., 2011). When rats are subjected to restricted feeding schedules, the daily rhythm of vasopressin release is shifted in the SCN (Kalsbeek et al., 1998) and neuropeptide Y (NPY) release in the paraventricular nucleus peaks in anticipation of food (Yoshihara et al., 1996). Moreover, studies have revealed that when rodents demonstrate food anticipatory behaviour a few hours before mealtime, hypothalamic and brain stem nuclei relevant for energy balance regulation increase their neuronal activity (measured as an increased c-Fos expression) (Angeles-Castellanos et al., 2004; Angeles-Castellanos et al., 2005). Interestingly, in the restricted feeding paradigm, food anticipatory activity in rats also leads to a peak in c-Fos expression in corticolimbic structures associated with motivation and reward (Angeles-Castellanos et al., 2007). Besides c-Fos expression, the expression of *Per1* and *Per2* was also altered by restricted feeding in these brain regions therewith detaching them from the SCN input signal reward (Angeles-Castellanos et al., 2007). Dysregulating effects of restricted feeding during daytime on the rhythmic expression of clock-genes have been found in several additional brain regions such as the hippocampus, prefrontal cortex, striatum, nucleus accumbens, olfactory bulb, and several forebrain structures involved in stress regulation, motivation, emotion, and ingestive behaviours. Notably, differential effects (i.e., increased or decreased and shifted rhythmicity in gene expression) have been found depending on brain region (see **Figure 4**) (Escobar et al., 2011). Studies in mice have shown that independent of caloric restriction, locomotor rhythms shift towards the time of day a highly palatable snack (meaning highly pleasant-tasting) is provided (Escobar et al., 2011; DePoy et al., 2017). For example, presenting male CBA/CaJ mice with chocolate every day at noon led to increased daytime activity, measured as running wheel activity highlighting timing of food as a factor that can alter the daily allocation of rest and activity (van der Vinne et al., 2015). This notion is particularly interesting as it might relate to our own, human, cravings for rewarding snacks that vary throughout the day and in line with our cognitive performance (Albrecht, 2011). Along these lines, a specific snack consumed at a fixed time in the afternoon might alter activity and/or performance levels. An early review article (Kanarek, 1997) shed some light on the psychological effects of snacks and meal frequency, for example concluding that nutrient intake later in the afternoon appears to facilitate subsequent performance on tasks involving sustained attention or memory. However, a number of factors have to be considered when assessing the effect of meal intake on behaviour, e.g., objective parameters such as nutrient composition or meal size, as well as individual factors such as age and gender, general activity level, or personality factors and subjective (snack) preferences (Kanarek, 1997).

Of note, the impact of subjective preferences for snack rewards on neural reward processing has recently been investigated in a series of studies with electroencephalography (EEG). These studies found that subjective preferences are complementarily represented in subjective reward valuation and in motivational value representations as reflected by distinct components of the event-related potential (ERP) (Peterburs et al., 2019b; Huvermann et al., 2021). Importantly, reward processing was not only modulated by individual factors such as subjective preferences for snacks (e.g., if one prefers salty or sweet snacks), but also by the current motivational state (e.g., the individual craving for snacks), since selective devaluation of a high preference snack by consumption to satiety decreased reward-related ERP activity (Peterburs et al., 2019b; Huvermann et al., 2021). Unfortunately, these studies did not assess

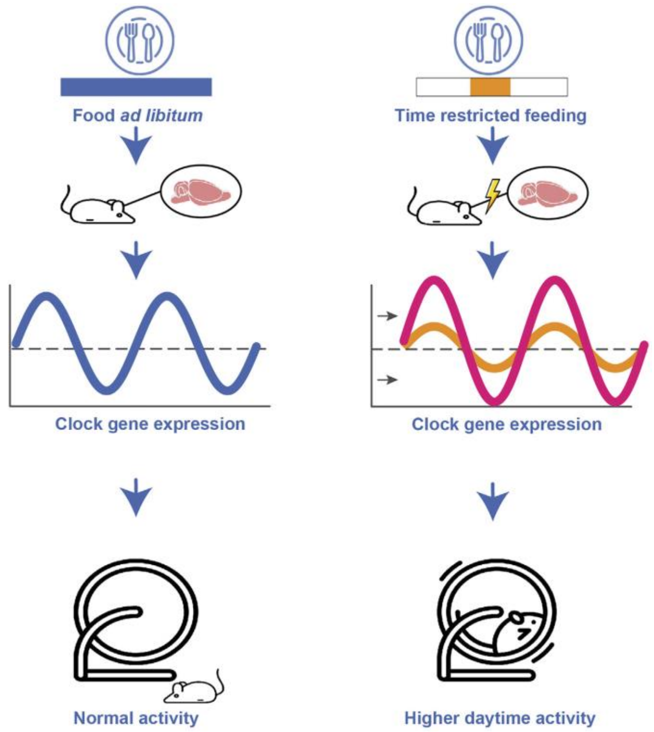

FIGURE 4 | Schematic representation of the impact of altered food schedules on the circadian clock. Restricted feeding during daytime leads to a shifted, attenuated or increased clock gene expression depending on brain region. Furthermore, restricted feeding during daytime results in higher daytime activity compared to the normally low daytime activity.

circadian influences in reward processing as a function of meal intake or food preferences, nor did they acquire any measure of (cognitive) performance. However, other work has hypothesized a connection on the molecular level between circadian clock, reward system, and cognitive performance, here specifically memory (Albrecht, 2011). Circadian clock genes regulate the synthesis, function, and degradation of dopamine via specific processes (thus directly influencing monoaminergic signalling in areas of reward processing), and might thereby affect reward and reward-related behaviour (DePoy et al., 2017).

Circadian Rhythm and Cognitive Performance

As above-mentioned, cognition is also affected by circadian rhythmicity, with effects found e.g., for attention, working memory, and executive functions, showing that performance is typically increasing during the day and decreasing during night (reviewed by (Valdez et al., 2012; Valdez, 2019)). For example, in humans, attention reaches the lowest level during night time and the early morning hours, shows better levels around noon, and the highest levels in the afternoon and evening (Valdez, 2019). Accordingly, cognitive performance can be influenced by chronotype, age, and sleep deprivation (Valdez, 2019).

On the hormonal level, melatonin might act as modulator of cognitive functioning and memory processing. Animal studies have provided evidence that melatonin decreases or inhibits long-term potentiation in the hippocampus (Wang et al., 2005; Bob and Fedor-Freybergh, 2008; Rawashdeh and Maronde, 2012). Findings is humans are less clear (Killgore et al., 2018; Rawashdeh et al., 2018). A neuroimaging study in healthy subjects did point to an effect of morning melatonin on working memory processes (Killgore et al., 2018). A decline in melatonin in the morning was associated with increased prefrontal cortex functioning. However, this effect concerned vigilance performance rather than cognitive load-dependent working memory performance. Gorfine *et al.* (Gorfine et al., 2007) investigated performance in a verbal memory task performed 2 hours after melatonin or placebo intake and failed to find differences.

Of note, clinical research in humans mainly focuses on potentially beneficial effects of melatonin treatment on cognitive and/or memory function. For instance, a recent systematic review and meta-analysis found that melatonin treatment significantly improved cognitive scores in mild levels of AD (Sumsuzzman et al., 2021). Again, effects on healthy individuals were less clear.

Cerebellum and Circadian Rhythm

Besides brain areas underlying reward processing or memory formation and consolidation, another interesting brain structure in the context of circadian rhythms is the cerebellum. The cerebellum has traditionally been thought to primarily orchestrate motor learning and the coordination of movements as well as the timing of muscle groups to assure fluid movements (Manto et al., 2012). However, a paradigm shift has led to emphasis also on cerebellar involvement in non-motor, cognitive functions such as working memory (Desmond et al., 1997; Peterburs et al., 2019a; Peterburs et al., 2021), language and verbal fluency (Ackermann et al., 2007; Peterburs et al., 2010), error and feedback processing (Peterburs et al., 2012; Peterburs et al., 2015; Peterburs et al., 2018), and performance monitoring in general (Peterburs and Desmond, 2016).

Studies in mice have revealed that the cerebellum, like other brain regions, has a circadian oscillator relevant for the anticipation of food (Mendoza et al., 2010). The researchers analysed the effects of food restriction on the expression of clock genes in the cerebellum as well as food-anticipatory behaviours in mice with impaired cerebellar functioning (Mendoza et al., 2010). First, restricting access to food resulted in phase-shifting effects on clock gene expression in healthy mice. Second, the group investigated whether impaired cerebellar circuitry, resulting from either intracerebroventricular injection of an immunotoxin depleting Purkinje cells or from a genetic mutation (leading to impaired cerebellar circuitry and mild ataxia) changed food-anticipatory behaviours. Interestingly, mice with impaired cerebellar circuity showed reduced to no regular food-anticipatory behaviour and altered clock gene expression (Mendoza et al., 2010). These findings point towards a functional cerebellar oscillator as part of the circadian network that is crucial for mealtime anticipation (Mendoza et al., 2010). To strengthen the circadian function of the cerebellum, researchers analysed the cerebellar proteome of mice regarding rhythmic expression patterns and revealed that most proteins in the cerebellum show a rhythmical expression leading to a bimodal distribution (midday and midnight) (Plumel et al., 2019).

Another relevant aspect in the context of circadian rhythmicity is the connection between the cerebellum and the sleep-wake cycle. The cerebellum shows sleep stage-dependent activity, with disruptions changing the sleep-wake cycle (Canto et al., 2017). These changes thus can result in sleep disorders such as chronic insomnia, fatal familial insomnia, or obstructive sleep apnoea or REM sleep behaviour disorder. In line with this, sleep disorders have been associated with reduced cerebellar volume, and patients with cerebellar dysfunctions frequently suffer from sleep problems (Canto et al., 2017).

Circadian Clock Disruption and Mental Disorders

Generally, mental disorders have been associated with a variety of alterations on the neuronal level such as volumetric reductions and neuronal structural properties (Bas-Hoogendam et al., 2020; Mundorf et al., 2021d), atypical hemispheric asymmetries (Mundorf et al., 2021c), atypical regional morphometry (Bas-Hoogendam et al., 2017), and altered neuronal gene expression in rodent animal models of mental disorders such as addiction, schizophrenia and mood disorders (Mundorf et al., 2020; Mundorf et al., 2021a; Mundorf et al., 2021b). Another aspect common to most mental disorders is a disruption of circadian rhythms, which has been investigated extensively in both animal models and clinical samples. The following paragraphs will focus on clinical research in circadian disruptions.

Individuals suffering from depression, OCD, attention deficit hyperactivity disorder (ADHD), or dementia show pronounced alterations in circadian rhythmicity in the sleep/wake cycle (also insomnia), reduced melatonin concentrations, and associated impairments in cognitive performance (Valdez et al., 2012; Valdez, 2019). Moreover, most of the prescribed medications for the respective disorders not only improve cognitive performance and affect but also influence circadian rhythms (e.g., by suppressing circadian rhythms of sleep/wake cycle, or increasing sleepiness) (Valdez et al., 2012).

In MDD and Bipolar Disorder (BD), circadian clock alterations are indicated by sleep disruptions, which represent a core/cardinal symptom and diagnostic criterion (Joel et al., 2015). In MDD, circadian clock disruptions have been studied at the molecular, cellular, physiological, and behavioural level. For example, post-mortem studies provide preliminary evidence that–in comparison to healthy control subjects–melatonin 1 receptors in the SCN and SCN GABA expression (**Figure 2A**) are heightened, and further that expression patterns of circadian genes in limbic and cortical brain regions are weakened in depressed patients (Li et al., 2013; Wu et al., 2013; Wu et al., 2017).

Circadian rhythms have also been investigated in the context of management of disorder management. Common treatments include total sleep deprivation, a protocol in which the patients spend 1 day (or more) without sleep to reset their sleep schedules. Results were further improved when combined with a follow-up treatment using BLT or a short phase advance protocol in several disorders (Echizenya et al., 2013), including MDD (Wirz-Justice, 2006; McClung, 2007; Kundermann et al., 2008) and Seasonal Affective Disorder (SAD) (Lam et al., 2006; Campbell et al., 2017). In meta-analyses on genetic studies, however, associations between clock genes including *CLOCK, CRY1, CRY2, PER2, NPAS2* in MDD could not be confirmed (Kishi et al., 2011; Melhuish Beaupre et al., 2020). Moreover, patients with MDD show disrupted sleep wake cycles (Germain and Kupfer, 2008), altered melatonin secretion patterns (De Berardis et al., 2015), higher nocturnal body temperatures, and decreased 24 h body temperature amplitudes when compared to healthy control subjects (Avery et al., 1982; Avery et al., 1986; Avery et al., 1999; Lorenz et al., 2019). Findings on physical activity are controversial. While some studies report preliminary evidence for lower physical activity (Difrancesco et al., 2019) and a negative correlation between depressive symptom severity and physical activity in depressed individuals (Difrancesco et al., 2021), another study found no such effects (Lorenz et al., 2019).

Like in MDD, circadian clock disruptions have been widely studied in BD. Genetic studies suggest associations between certain core clock genes such as *CLOCK, ARNTL, NPAS2, PER3* and *NR1D1* and BD–however, findings still require replication (Etain et al., 2011). Beyond that, there is preliminary evidence that melatonin profiles and *PER1* and *NR1D1* expression profiles of manic patients with BD differ from those of depressed BD patients and healthy controls (Nováková et al., 2015). Meta-analyses on circadian physiological and behavioural processes show differences between (remitted) patients with BD and healthy control subjects in total sleep time and time in bed, sleep latency, wake after sleep onset and motor activity (Geoffroy et al., 2015; Ng et al., 2015; Nováková et al., 2015; De Crescenzo et al., 2017; Meyer et al., 2020).

While most of the literature has focused on affective disorders, there is also some evidence for sleep disruptions in OCD (Coles and Stewart, 2019; Sevilla-Cermeño et al., 2019; Coles et al., 2020). To our knowledge, however, there are currently no existing studies investigating circadian clock disruptions in OCD at the molecular or cellular level. Only recently, one genetic study found an association between certain clock-controlled genes, e.g. *ARNTL* rs2278749 polymorphism, with obsessive-compulsive symptoms in a healthy control sample (Jeong et al., 2019). Moreover, findings from studies on circadian hormonal rhythms (cortisol and melatonin secretion) in OCD are still controversial regarding differences between patients and healthy control subjects (Catapano et al., 1992; Millet et al., 1999; Kluge et al., 2007). However, several meta-analyses show differences between patients with OCD and healthy control subjects in total sleep time, time awake, sleep efficiency, sleep latency, and variability in REM sleep parameters (Díaz-Román et al., 2015; Nota et al., 2015; Cox and Olatunji, 2020), strongly suggesting that circadian rhythms should be further studied in OCD.

Circadian clock disruption is also common to psychotic conditions such as schizophrenia and schizophrenia-spectrum disorders (Ashton and Jagannath, 2020). In a small sample of eleven patients with chronic schizophrenia, the expression of the clock genes *CRY1* and *PER2* has been shown to differ in comparison to healthy control subjects (Johansson et al., 2016). Beyond that, circadian gene expression appeared to be less rhythmic in patients with schizophrenia compared to healthy control subjects (Seney et al., 2019). Further, a recent meta-analysis, reported that non-acute patients with schizophrenia-spectrum disorders differ from healthy control subjects in total sleep time, time in bed, sleep latency, wake after sleep onset, and motor activity (Meyer et al., 2020).

Circadian abnormalities in schizophrenia have been hypothesized to be related to dysregulations in multiple neurotransmitter systems including hyperactivity of the dopaminergic and dysfunction of the GABAergic system (for a comprehensive review, see Monti et al., 2013). Dopaminergic dysregulation has also been linked to acceleration of the internal clock even outside of circadian effects (Davis et al., 1991), as evidenced by alterations of interval timing and duration estimation (e.g., Meck, 1996). In a study by Peterburs et al. (2013), administration of the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) allowed assessment of individual levels of positive and negative symptoms. When asked to estimate the time needed for a moving visual stimulus to reach a specific target position, patients underestimated movement time. This was especially prominent in patients with high PANSS positive symptoms (Peterburs et al., 2013).

It must be noted that time processing anomalies in schizophrenia such as altered interval timing or duration estimation have been investigated mostly independently from circadian rhythms. However, some researchers have argued that it is reasonable to assume that these processes may share some of their underlying mechanisms. For instance, clock genes may affect interval timing via neural oscillator networks which are mediated by dopaminergic signalling or alterations of neuronal architecture or excitability (Nicholas, 2015).

Taken together, evidence of disturbances of the circadian clock appear to occur in many mental disorders, yet the precise understanding of these alterations is currently missing, which precludes informing treatment strategies. Notably, some conditions (e.g., OCD) have been studied less extensively than others (e.g., MDD, BD). Possibly, this is due to the fact that circadian disturbances are closely linked to core symptoms only in the latter (i.e., sleep disturbances). Yet consistent evidence of sleep disturbances in conditions such as OCD indicates that a clear characterization of circadian clock alterations could also prove to be beneficial for treatment strategies in disorders in which the link between circadian clock disruption and clinical symptoms is less obvious. A goal for future research is thus to close gaps in knowledge on circadian clock disruptions in mental disorders on the molecular, cellular, physiological and behavioural level and to unravel the associated aetiology, in order to identify new starting points for prevention and early intervention.

CIRCADIAN RHYTHMS, PHYSICAL EXERCISE AND COGNITIVE PROCESSES

The human brain remains in constant change, altering its functional and structural properties to adapt to changing demands (Budde et al., 2016b). This intrinsic characteristic of our brain is called neuroplasticity and allows the nervous system to adapt to environmental pressures, physiologic changes, and experiences by escaping the restrictions of its own genome (Pascual-Leone et al., 2005). Findings have shown a dynamic remodelling of grey matter throughout life characterized by the continuous creation and growth of neurons, dendrites, and new synapses as well as their elimination (Killgore et al., 2013). This plastic capacity of the brain represents the normal constant state of the nervous system through the life cycle, and is understood as a human mechanism for learning (e.g., the asset of new skills

(Hötting and Röder, 2013)) growth, and general development but it could also be a cause of pathology (Pascual-Leone et al., 2005).

The natural process of aging is known to lead to functional changes in our brain that usually translates to functional and social impairments and a general cognitive decline (Bettio et al., 2017). At a cellular level, our brain plasticity is reduced as we age. There is a decrease in the creation of new synapses (Heine et al., 2004), and on average, synapses make fewer contacts, with less strength than in previous stages of life (van Praag et al., 2005). Neurobiological alterations like an increase of oxidative stress and neuroinflammation, as well as distorted intracellular signalling and gene expression, have also been associated with age-related cognitive decline (Bettio et al., 2017). However, the number of synapses, as well as the changes in their morphology, seem to oscillate not only on a long-term basis, but also on a daily cycle basis. These approximately 24 h cyclic changes in the nervous system are attributed to the circadian clock and referred to as circadian plasticity (Krzeptowski et al., 2018). Dysregulation of this system seems to affect major brain functions and may cause nervous system disorders (Logan and Colleen, 2019).

It is known that developmental changes are highly influenced by genetic factors. Over the last decades, interest in the study of the circadian typology has grown notably. Findings have pointed to individual differences on circadian rhythmic expression (of genes, hormones or biological processes such sleep or body temperature variations) affecting biological and psychological functioning in health and disease (Adan et al., 2012). Time-of-day fluctuations seem to play a role not only in cognitive functioning (Schmidt et al., 2007), but also in more specific areas such as physical performance (Drust et al., 2005).

As described in detail in **Sections 3.2**, in addition to governing sleep-wake cycles, circadian rhythms govern rhythms in cognitive processes like subjective alertness, mathematical ability, memory processes, and attention (Benca et al., 2009; Valdez, 2019). Executive functions (EF) are high-level cognitive processes with an essential role in the regulation of information traffic (Diamond, 2013). These functions enable us to generate the necessary sequences of actions needed to elicit the correct response to specific internal and external stimuli demands (Amatriain-Fernández et al., 2021) allowing, for example, to mentally play with ideas, to be adaptable to changing circumstances, to think before acting, to resist temptations, to stay focused or to be able to face new and unforeseen challenges (Diamond, 2013). There is general agreement that there are three core EF: inhibition, working memory, and cognitive flexibility (Diamond, 2013). It is known that EF suffer a decrease in their capacity to function due to developmental changes and ageing (Ferguson et al., 2021). However, external circumstances like stress, lack of sleep, loneliness, or lack of exercise seem to also affect these functions (Diamond, 2013).

Findings have shown that people with decreased circadian activity rhythm amplitude (peak activity) and robustness, as well as people with delayed rhythms have higher probabilities of developing dementia and mild cognitive impairment (Tranah et al., 2011). Along these lines, proper maintenance of the 24 h clock seems highly relevant for regulating processes such as neural activity, or hormonal (like the cortisol awakening response) signalling that may influence the development of disorders of the central nervous system (Logan and Colleen, 2019), as well as metabolic health (van Moorsel et al., 2016). Besides, disruptions of sleep and circadian rhythms are among the most debilitating symptoms in patients that already have neurodegenerative diseases (Fifel and Videnovic, 2021).

Aside from genetic influences, non-genetic factors such as physical exercise seem to also benefit the organization and development of some cerebral systems (Killgore et al., 2013), and even to adjust the circadian rhythm to external time cues (Yamanaka et al., 2006).

The terms physical exercise and physical activity are often used interchangeably. Nevertheless, while physical activity is defined as any bodily movement produced by the contraction of skeletal muscles that results in a substantial increase in caloric requirements over resting energy expenditure (American College of Sports Medicine, 2013), physical exercise is defined as a planned, structured, repetitive, and purposeful training exercise intervention that leads to a change in fitness (Budde et al., 2016a). Therefore, physical exercise is always a physical activity, but physical activity is not necessarily always physical exercise (Wegner et al., 2020). Exercise interventions are usually differentiated based on parameters like length, intensity, or type of exercise. While chronic interventions contain several bouts of exercise over a long period of time, acute interventions include a single bout of exercise, performed only once (Amatriain-Fernández et al., 2021). Consequently, chronic interventions can be used to evaluate the long-term effects of an exercise intervention and acute interventions are implemented to assess the immediate effects of an exercise intervention (Amatriain-Fernández et al., 2021).

Physical Exercise and Cognitive Processes

Evidence from both animal and human studies indicates that physical exercise facilitates neuroplasticity of certain brain structures (Budde et al., 2016b). Studies with animals models (mice and rats) identified several neural mechanisms that might mediate the beneficial cognitive effects of physical exercise: the enhancement of neurogenesis, synaptogenesis, angiogenesis, and the release of neurotrophins (Hötting and Röder, 2013), like the brain-derived neurotrophic factor (BDNF), with an crucial role for synaptic plasticity, learning and memory (Vaynman et al., 2004). Besides, in the dentate gyrus of the hippocampal formation, increased blood flow and oxygenation seem to correlate with exercise-induced neurogenesis (Pereira et al., 2007). Following voluntary wheel running, increased neurogenesis in the dentate gyrus has been detected in adult mice (van Praag et al., 1999; Brown et al., 2003; van Praag et al., 2005; Kronenberg et al., 2006). Neurons generated under the running condition, were found to be functionally integrated into the hippocampal circuitry and indistinguishable from mature cells in their electrophysiological properties (van Praag et al., 2002). Following different exercise interventions that included running, adult mice showed an enhanced memory consolidation (Kohman et al., 2012) and adult rats showed a better memory abilities like learning and recalling the location of a specific platform (Vaynman et al., 2004). In adolescent rats, regular moderate aerobic treadmill exercise resulted in improvements in spatial learning abilities and an exercise-induced increase of cell density in the hippocampus and dentate gyrus (Uysal et al., 2005).

In humans, findings appear to have a similar trajectory. It has been suggested that physical exercise may trigger several processes facilitating neuroplasticity, increasing the human capacity to respond to new demands with behavioural adaptations (e.g., the allocation of greater attentional resources, the ability to process information quicker or an enhanced memory performance) (Hötting and Röder, 2013). Even though the underlying mechanisms associated with exercise-induced in the human brain remain to be elucidated (Killgore et al., 2013), some advances have been made in recent years. Exercise is thought to activate the necessary transcriptional machinery to modulate the expression of genes related to the regulation of synaptic plasticity, learning and memory using epigenetic mechanisms (Fernandes et al., 2017). This is not only relevant for neural cell differentiation, but also for experience-induced changes in human brain function and behaviour (high-order cognitive functions) (Feng et al., 2007). Physical exercise seems to modulate several factors relevant for the connection between skeletal muscle and the brain, such as neurotrophins (e.g., BDNF and plasma Cathepsin B (CTSB) levels) and oxidative stress parameters (e.g., lipid oxidation markers) that help to mitigate cognitive decline (De la Rosa et al., 2019). In this regard, recent research situates physical exercise as a mediator of neurogenesis in the hippocampus via BDNF (Liu and Nusslock, 2018). Exercise interventions have also shown to produce benefits in the physical functioning, health-related quality of life, strength, balance, and gait speed of PD patients (Goodwin et al., 2008). Recent findings have suggested that exercise improves sleep quality in people with Parkinson (Amara et al., 2020), situating it as an effective nonpharmacological intervention to improve this disabling nonmotor symptom that normally comes with the disorder.

Results from longitudinal studies have also shown that long-term physical exercise interventions (35 ± 15 years) promoted memory maintenance in middle-aged men and showed a connection between delay of age-related neurodegeneration and the neurotrophic and redox peripheral modulation produced by the physical exercise intervention (De la Rosa et al., 2019). Lower cerebral blood flow was associated with poorer memory and processing speed abilities in older adults with type 2 diabetes (Bangen et al., 2018) as well as with poorer attentional and memory capacities in adults with cardiovascular disease (Alosco et al., 2013).

Exercise has been situated as a preventive factor for age-related cognitive decline (Kronenberg et al., 2006), but the positive effects of exercise interventions on cognition are not limited to elderlies with preconditions. Findings showed that, in general, older adults who exercised throughout life exhibited less brain tissue loss than their sedentary peers (Colcombe and Kramer, 2003). Aerobic exercise interventions have produced increased hippocampal volume and enhanced cognitive functioning in different age-samples (Killgore et al., 2013). A study with overweight children found that increased cardiorespiratory fitness and speed-agility may have a positive impact on the development of different regions in the brain as well as on academic indicators and might neutralize the harmful effect caused by obesity and overweight on brain structures (Esteban-Cornejo et al., 2017). Additionally, a study with pre-adolescents showed that those with higher physical fitness levels presented greater grey matter volume in the hippocampus and performed better on cognitive tests than peers with lower fitness level (Chaddock et al., 2010). Similar results were obtained for healthy adults (Killgore et al., 2013). Further, effects of exercise seem to be longer lasting than previously thought which situates exercise as an epigenetic modulator of brain plasticity and cognition (Fernandes et al., 2017).

Physical Exercise and Circadian Rhythms

Both, animal (Yamanaka et al., 2008; Wolff and Esser, 2012) and human studies (Yamanaka et al., 2006; Okamoto et al., 2013; Basti et al., 2021) have also found that exercise can alter circadian rhythms in behaviour and gene expression. In addition, the circadian clock seems to also have an influence on the benefits of exercise interventions pertaining to cognitive and physical performance (Atkinson and Reilly, 1996; Drust et al., 2005; Waterhouse et al., 2005; Facer-Childs and Brandstaetter, 2015b; a; Facer-Childs et al., 2018).

Studies on this topic have reported that circadian fluctuations in molecular (gene expression) and physiological (biomechanical muscle properties) parameters correlated with exercise performance (Basti et al., 2021). In a study comparing rugby players with sedentary subjects, it was found that genes related to circadian rhythms (*BMAL1*, *ROR-α*, *CRY1*, *PER2* ($p < 0.001$), *PER1* ($p < 0.01$, and *NR1D1* ($p < 0.05$) were higher in rugby players, indicating that long-term exercise can increase the expression of genes related to the circadian clock. Further, exercise performance has shown peaks in the late afternoon (15–18 h) for healthy men and women (Basti et al., 2021). These authors reported that the daily fluctuations exhibited different patterns depending on the type of exercise (endurance vs. strength) and were also accompanied by fluctuation on the expression of core-clock genes and muscle tone (Basti et al., 2021).

Differences in performance have also been investigated in relation to the individual chronotype. A study by Facer-Childs and colleagues reported that cognitive and physical performance showed a significant diurnal variation when comparing early and late chronotypes. In this study 56 healthy individuals were categorized as early or late chronotypes based on the Munich Chronotype Questionnaire (MCTQ), and their performance in tasks related to psychomotor vigilance, executive functions, and isometric grip strength showed significant variations along the day. Late chronotypes performed significantly poorer, during the morning, than early chronotypes (Facer-Childs et al., 2018). In line with these results, evening-type swimmers swam 6% slower in the morning than in the evening trial, while morning-type swimmers required 5–7 times more effort to achieve the same performance results in an evening trial as obtained in a morning trial (Anderson et al., 2018). These authors established the chronotype based on the MCTQ. Their diurnal preference was assessed by the self-reported Hornestberg Morningness-Eveningness Questionnaire (MEQ) and strands of hair were collected to characterize the genotype of each participant for PER3 SNP rs228697 and PER3 VNTR rs57875989.

In sum, despite the need for more research to deep into the connections between physical exercise and circadian rhythms, the literature points to a bidirectional relation between circadian rhythms and exercise. Exercise may help to ameliorate circadian dysregulations and circadian rhythms seem to affect physical performance.

EPIDEMIOLOGICAL EVIDENCE TOWARDS CIRCADIAN REGULATION OF MENTAL HEALTH

Epidemiological studies have established the role of the circadian clock and circadian misalignment as a factor in physical and mental health. The term circadian misalignment traditionally refers to a mismatch between an individual's circadian chronotype and the physical or social environment (e.g., light-dark cycle, school, or work times) (Roenneberg and Merrow, 2016). In the general population, "social jet lag" represents a proposed cause of circadian misalignment. Social jet lag refers to the discrepancy between the duration of sleep during work days and non-work days (e.g., weekends) (Wittmann et al., 2006). The social Zeitgeber Theory associated with mental disorders posits that stressful life events may give rise to alterations of circadian dynamics at the cellular and physiological levels (e.g., disrupted sleep-wake cycles). As a result, affected individuals are more prone to mood-related incidents (Ehlers et al., 1988; McClung, 2013). Epidemiological studies further highlighted the common nature of social jet lag, indicating that most individuals demonstrate variations in wake-sleep times between workdays and free days, with up to 87% of the day-working population suffering from social jet lag (Roenneberg et al., 2003; Roenneberg et al., 2007; Roenneberg et al., 2012). For instance, discrepancies between the circadian chronotype and the socially determined opportunity for sleep can arise when 'normal' chronotypes are employed in shift work schedules or when extreme chronotypes have to comply with conventional work hours. Extreme chronotypes (i.e., preference for either early or late sleep/activity) may evolve from a combination of genetic predisposition, age, and Zeitgeber conditions (Carskadon et al., 2004; Roenneberg et al., 2004; Roenneberg et al., 2013b; Hsu et al., 2015). The need to conform to conventional work times makes evening chronotypes (i.e., preference for later sleep/activity) more susceptible to social jet lag (Duffy et al., 2001; Mongrain et al., 2006).

Besides the immediate repercussions, which pertain to sleep disturbance and/or daytime sleepiness, social jet lag has been linked to metabolic functioning, smoking, alcohol and caffeine consumption (Wittmann et al., 2010; Roenneberg et al., 2012), as well as an increased risk for depression (Drennan et al., 1991; Chelminski et al., 1999; Gaspar-Barba et al., 2009; Kim et al., 2010; Kitamura et al., 2010; Levandovski et al., 2011; Merikanto et al., 2013). Although epidemiological research has demonstrated that in the general population, chronotype follows a normal distribution varying by gender and age, societal changes such as the increase in shift work contribute to circadian misalignment and sleep disruptions. To this end, the majority of studies has incorporated methodologies focused on shift work and jet lag, utilizing questionnaire (i.e., self-report) data rather than genetic or molecular profiling to determine chronotypes.

Over the last decades, societal changes have sparked an increase in shift work with currently at least 20% of the population's employment based on shift work schedules (Baron and Reid, 2014). Shift work typically refers to work schedules that greatly overlap with primary sleep times, i.e., where at least 50% of work falls between 10 p.m. and 6 a.m. (Baron and Reid, 2014). Compared to non-shift workers, night and early morning workers (i.e., start before 6 a.m.) experience significant sleep time reductions of between 1 and 4 h per day (Knauth et al., 1980; Åkerstedt, 1998). As a consequence, shift work is linked to reduced sleep duration and sleep quality (Drake et al., 2004). Further, associations between shift work and various negative physical and mental health outcomes, such as cancer, cardiovascular disease, obesity, diabetes, reproductive health complications, and memory difficulties have been established (Nurminen, 1998; Labyak et al., 2002; Knutsson, 2003; Drake et al., 2004; Gumenyuk et al., 2010; Esquirol et al., 2011; Gumenyuk et al., 2014; Ohlander et al., 2015; Reutrakul and Knutson, 2015; Wong et al., 2015). These adverse health outcomes may be attributed to a complex combination of maladaptive health behaviours (e.g., smoking, alcohol consumption, healthy food restrictions, feeding patterns), chronic sleep deprivation, circadian misalignment, and increased nocturnal light exposure (Baron and Reid, 2014; Abbott et al., 2015).

A hypothesized mechanism behind the link between shift work and cancer in particular has been exposure to light at night (LAN). According to the LAN hypothesis, reduced levels of the endogenous hormone melatonin may be at the centre of this link. Because melatonin is primarily produced at night and sensitive to light suppression, the LAN hypothesis proposes that the transformation of normal cells into cancer cells (i.e., carcinogenesis, oncogenesis) is reinforced by exposure to light at night. Although a clear dissection of cause and effect is difficult given the complex impact of modern lifestyles and the fact that circadian misalignment is associated with many pathologies unrelated to melatonin, epidemiological evidence for the link between LAN and cancer is strong (Hansen, 2001; Schernhammer et al., 2001; Schernhammer et al., 2006; Flynn-Evans et al., 2009; Grundy et al., 2013; Hurley et al., 2014). In fact, evidence in this area provoked the World Health Organization (WHO) to include shift work as a potential carcinogen in 2007 (WHO, 2011).

Given the aforementioned impact of the circadian rhythm and misalignment thereof on mood and interpersonal behaviours, it is almost surprising that only a limited amount of research has focused on the role of circadian rhythm during the transition to parenthood. It is by now well recognized that the perinatal period (i.e., from pregnancy to around 12 months following birth) marks a time of high vulnerability for mental health complications for (expectant) parents, with adverse outcomes for mothers, fathers, and their offspring (Thiel et al., 2021b; Knappe et al., 2021). In the first weeks following birth, for instance, up to 85% of women will experience deteriorations in mood. This so-called "postpartum blues" has been identified as a predictor of postpartum depression (Beck, 1996), which affects around 13–19% of women (O'Hara and Swain, 1996; Gavin et al., 2005) and has been documented as the most frequent complication of childbirth (Moses-Kolko and Roth, 2004; Moore Simas et al., 2019). The majority of research on parental perinatal mental health has focused on symptoms of depression (O'Hara and Swain, 1996; Eberhard-Gran et al., 2003; Paulson and Bazemore, 2010; Junge et al., 2017; Garthus-Niegel et al., 2020; Thiel et al., 2020b), anxiety (Eberhard-Gran et al., 2003; Polte et al., 2019; Thiel et al., 2020a), and post-traumatic stress disorder (Garthus-Niegel et al., 2013; Garthus-Niegel et al., 2017; Garthus-Niegel et al., 2018; Thiel et al., 2018; Dekel et al., 2019; Thiel and Dekel, 2020; Thiel et al., 2021a; Kress et al., 2021). Whereas initially, maternal mental health was at the core of this research area, increasing attention has been paid to fathers'/ partners' perinatal mental health as well (Kress et al., 2019; Thiel et al., 2020b; Garthus-Niegel et al., 2020; Kress et al., 2021; Asselmann et al., 2022a; Asselmann et al., 2022b).

During pregnancy, alterations in women's sleep patterns and quality with adverse impacts on mood have been documented to begin as early as during the first trimester (Ross et al., 2005; Bei et al., 2015). Over the course of pregnancy, sleep duration and efficiency decrease and sleep quality is further reduced (Hedman et al., 2002; Adler et al., 2021). Further, over 40% of women experience insomnia during the first trimester of pregnancy, increasing to over 60% by the third trimester (Román-Gálvez et al., 2018). Following birth, self-reported deteriorations in sleep quality, efficiency, and duration continue for both, mothers and fathers (Lee et al., 2000; Kang et al., 2002; Montgomery-Downs et al., 2013; Obeysekare et al., 2020). Compared to the time before pregnancy, daily sleep duration has been found to be reduced by 62 min for women and 13 min for men during the first 3 months following birth (Richter et al., 2019). Importantly, deteriorations in sleep quality and duration with onset during the transition to parenthood may not fully recover for years following birth (Lee et al., 2000; Richter et al., 2019).

The potentially crucial role of sleep disruptions during the transition to parenthood in mental health may be of interest for instance with regards to postpartum psychosis, which affects 0.1–0.2% of women following childbirth. Because around 20% of women with a history of bipolar disorder experience manic episodes, which often characterize postpartum psychosis, and because those experiencing postpartum psychosis frequently continue to report symptoms of bipolar disorder later on, it has been proposed that postpartum psychosis may be a manifestation of bipolar disorder triggered by childbirth (Ross et al., 2005; Lewis et al., 2016). Postpartum psychosis may be triggered by factors also related to mania, with disruptions of sleep and the circadian rhythm as the most common triggers being (Jackson et al., 2003; Lewis et al., 2016). Because symptoms of postpartum psychosis typically begin in the immediate perinatal period (Heron et al., 2008), the period with the greatest sleep disruption (Beebe and Lee, 2007), sleep and circadian rhythm disruptions may represent risk factors for the development of postpartum psychosis in those at risk (Lewis et al., 2016). Although the link between disruptions in sleep and circadian rhythm and postpartum psychosis is often hypothesized, empirical evidence is still limited. Nonetheless, a study frequently cited in this context by Sharma and colleagues documented that postpartum psychosis was linked to night deliveries and longer labor duration, pointing towards the crucial role of sleep disruption in the development of postpartum psychosis (Sharma et al., 2004).

Despite extensive investigation of parental sleep patterns and quality during pregnancy and following childbirth as well as its relationship with mental health complications (Deforges et al., 2021), only little is known about parental circadian rhythm during this time. Initial evidence suggests that parenthood is linked with an early chronotype (Sládek et al., 2020). Further, parental circadian rhythm amplitude may be decreased following birth (Wulff and Siegmund, 2000), which can be observed as a result of irregular sleep-wake patterns or disturbance and may accumulate to circadian rhythm sleep disorder (Zee and Vitiello, 2009). The primary decline in amplitude ensues immediately following birth as a result of infant care during the night. Longitudinal studies of women following birth document worsening of amplitude reduction during the first postpartum weeks (Nishihara et al., 2002; Matsumoto et al., 2003). Although research pertaining to circadian rhythm chronotypes in relation to mental health vulnerability during the transition to parenthood is scarce, initial evidence suggests that evening chronotypes may be at an increased risk for psychiatric symptoms (Obeysekare et al., 2020).

Given the toll of pregnancy and the transition to parenthood on sleep-wake patterns, sleep quality, and efficiency resulting from infant and child care needs, the perinatal period offers a wide range of avenues for future research pertaining to the role of the circadian rhythm in mental health. Besides the vulnerability for mental health complications, the transition to parenthood is characterized by changes regarding employment, work schedules, as well as work division among couples. Large epidemiological studies have been implemented to address these issues (e.g., Kress et al., 2019). Longitudinal, epidemiological investigations following couples and children from pregnancy throughout the years following birth will offer the opportunity to gain new insights into the link between the circadian rhythm and long-term mental health in both women and men, as well as into early mechanisms involved in the development and manifestation of the human circadian rhythm from the time of birth.

CONCLUSION AND PERSPECTIVES

Timely regulation of cellular, physiological and behavioural processes plays a fundamental role in the, often subtle, border between health and disease. In this review, we focused on the current knowledge regarding proper functioning of the circadian system in healthy individuals and the disruption of circadian regulation observed in numerous neurodegenerative and mental disorders.

Given the broad influence of circadian rhythmicity and circadian clock genes on multiple cognitive domains such as attention, memory, or reward processing, as well as natural factors affecting daily rhythms such as dietary schedule and the sleep/wake cycle, it is not surprising that disruptions in our internal clock can result in mental health problems. Indeed, epidemiological studies have established circadian misalignment as a prominent risk factor in physical and mental health, and highlight the risks associated to social jet lag, which affects the vast majority of the day-working population. Aside from the immediate repercussions, pertaining to sleep disturbance and/or daytime sleepiness, social jet lag has been linked to adverse health behaviours and other mental health complications. Societal changes such as the increase in shift work appear to exacerbate circadian misalignment and sleep disruptions.

Given these increasing environmental risk factors such as shift work, circadian misalignment, and personal meal schedules (e.g., eating high caloric meals late in the evening), the impact on mental health can no longer be disregarded. This is particularly relevant given the growing body of research, linking several mental disorders to disrupted circadian rhythmicity. On the genetic level, several studies have found altered gene expression of clock related genes in patients suffering from mental disorders, further underlining the important role of the internal clock for mental health. However, is has to be noted that even though the association between disrupted circadian rhythms and some mental disorders, especially mood disorders, is well investigated (Walker et al., 2020), this association remains to be disentangled for many other disorders. The usage of external zeitgebers (e.g., light or physical exercise) are a promising approach for enhancing and/or re-setting circadian rhythms. This seems to efficiently contribute to diminish deterioration of neurocognitive functioning.

Circadian rhythms have been shown to influence both cognitive and physical performance. Performing an analysis of circadian rhythms in the health domain could facilitate the design of highly personalized exercise interventions. These interventions could act as preventive mechanisms to delay the onset of disorders related to circadian disruption. This combined effort between research on exercise and circadian rhythms could have a positive impact on the benefits of exercise in the prevention and treatment of several mental disorders. However, a crucial point in administering the “exercise polypill” is the dosing and, in turn, the prescription of the physical intervention (Herold et al., 2020). The answer to questions like what type of exercise is the most influenced by circadian rhythms or what type of exercise is the best intervention for preventing or treating a determined pathology needs to be established (Gronwald et al., 2018; Gronwald and Budde, 2019; Gronwald et al., 2019; Gronwald et al., 2020; Herold et al., 2020). In future studies, the inclusion of a sham condition might help to elucidate some of these open questions (Budde et al., 2018).

Although the impact of circadian rhythm and its misalignment on mood and behaviour is well known, it is surprising that only a limited amount of research has focused on the role of circadian rhythm alterations during the transition to parenthood. For (expectant) parents, the perinatal period marks a time of, high vulnerability to mental health complications, as well as severe sleep disruptions. Despite extensive investigation of perinatal parental sleep patterns and quality and its relationship with mental health complications, only little is known about disrupted parental circadian rhythm during this time, with initial evidence suggesting that evening chronotypes may be at an increased risk for psychiatric symptoms (Obeysekare et al., 2020). The perinatal period thus offers a wide range of avenues for future research. Longitudinal, epidemiological investigations following couples from pregnancy throughout the years following birth will offer the opportunity to gain new insights into the link between the circadian rhythm and long-term mental health, as well as into early prenatal mechanisms involved in the development and manifestation of the human circadian rhythm.

Thus, a new area in the medical field, circadian medicine, is slowly emerging and time-of-day adapted therapies personalized to the patient’s circadian rhythm might provide better therapeutic outcomes than commonly applied therapies. However, clinical applications of circadian intervention remain limited due to obstacles such as a current lack of a sufficient number of studies, logistic difficulties in implementing circadian treatment regimens into clinical routines, and variations of results pertaining to the desired effect of chronotherapy in comparison to standard treatment due to demographic factors such as age or gender. To overcome these obstacles, larger cohorts and a more precise stratification of patients are needed before circadian medicine can be used in clinical practice. In this regard, the combination of genomics and physiological data holds great potential for the future direction of circadian medicine.

Investigating not only disorder-specific, but also symptom-specific alterations in the circadian clock might render new insights into the aetiology and implications of mental (and other) disorders and could set the grounds to improve mental health by considering the circadian rhythm. Although research in the circadian field has grown considerably over the past years, as pointed out in our review a discrepancy between basic chronobiology research and the usage of this knowledge in clinical practice, for maintenance of health and prevention and treatment of disease is obvious. By now we know that time matters, and it is about time that we use this knowledge.

AUTHOR CONTRIBUTIONS

Supervision: J-CB, HB, SG-N, JP, AR; Visualization: MY, AM; Computational analysis: MY; Writing reviewing and editing: MY, AM, FT, SA-F, IK, J-CB, HB, SG-N, JP, AR.

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GLOSSARY

AD Alzheimer's disease
ATHD Attention deficit Hyperactivity disorder
AMT Anticipation of movement task
AVP Arginine vasopressin
BD Bipolar disorder
BDNF Brain-derived neurotrophic factor
BLT Bright light therapy
BMAL1 Brain and muscle ARNT-like 1
CNS Central nervous system
CSM Composite scale of morningness
CLOCK Circadian locomotor output cycles protein Kaput
CRC Colorectal cancer
CTSB Plasma Cathepsin-B
DD Dark/Dark
DRD1 Dopamine receptor D1
EEG Electroencephalography
ERP Event-related potential
ESS Epworth sleepiness scale
FRP Free running period
HD Huntington's disease
HTT Huntingtin
KO Knockout
LAN Light at night
LD Light/dark
MDD Major depressive disorder
NPY Neuropeptide Y
NR1D1 Nuclear receptor subfamily 1 group D member 1
NR1D2 Nuclear receptor subfamily 1 group D member 2
OCD Obsessive compulsive disorder
PANSS Positive and negative syndrome scale
PD Parkinson's disease
PDSS-2 Parkinson's disease sleep scale 2 scores
REV-ERB α nuclear receptor subfamily 1 group D member 1
REV-ERBβ nuclear receptor subfamily 1 group D member 2
ROR Retinoid-related orphan receptors (RORs)
SAD Seasonal affective disorder
SCN Suprachiasmatic nucleus
SWPAQ Sleep wake pattern assessment questionnaire
TEMPS-A Temperament evaluation of Memphis, Pisa and San Diego-autoquestionnaire
TTFL Transcriptional and translational feedback loops
VIP Vasoactive intestinal peptide

10. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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11. List of Publications

(*) equal author contribution; **(#)** equal senior contribution; (IF) impact factor (from the year of publication)

1. **Yalçin, M.*,** El-Athman, R.*, Ouk, K., Priller, J., Relógio, A. (2020). Analysis of the circadian regulation of cancer hallmarks by a cross-platform study of colorectal cancer time-series data reveals an association with genes involved in Huntington's Disease. Cancers. (IF: 6.126)

2. Basti, A., Fior, R., **Yalçin, M.**, Póvoa, V., Astaburuaga, R., Li, Y., Naderi, J., Ferreira, M. G., Relógio, A. (2020). The Core-Clock Gene NR1D1 Impacts Cell Motility. Cancers (IF: 6.126)

3. Hesse, J., Malhan, D., **Yalçin, M.**, Aboumanify, O., Basti, A., Relógio, A. (2020). An Optimal Time for Treatment—Predicting Circadian Time by Machine Learning and Mathematical Modelling. Cancers. (IF: 6.126)

4. Li, Y., Basti, A., **Yalçin, M.,** Relógio, A. (2020). Circadian Dysregulation of the TGFβ/SMAD4 Pathway Modulates Metastatic Properties and Cell Fate Decisions in Pancreatic Cancer Cells. iScience. (IF:5.08)

5. Basti, A.*, **Yalçin, M.*,** Herms, D.*, Hesse, J., Aboumanify, O., Li, Y., Aretz, Z., Garmshausen, J., El-Athman, R., Hastermann, M., Blottner, D., Relógio, A. (2021). 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. (IF:3.14)

6. Gaspar, L., Hesse, J.*, **Yalçin, M*.**, Santos, B., Carvalhas-Almeida, C., Ferreira, M., Moita, J., Relógio, A.#, Cavadas, C.#, Álvaro, A. R.# (2021). Long-Term Continuous Positive Airway Pressure Treatment Ameliorates Biological Clock Disruptions in Obstructive Sleep Apnea. EBioMedicine. (IF:11.205)

7. **Yalçin, M.,** Malhan, D., Basti, A., Peralta, R., Ferreira, J., Relógio, A. (2021). 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. (IF:6.639)

8. **Yalçin, M.,** Mundorf, A., Amatriain-Fernández, S., Kalthoff, I., S., Beucke, J., Budde, H., Garthus-Niegel, S., Peterburs, J., Relógio, A. (2022). It's about time: the circadian network as a time-keeper for cognitive functioning, locomotor activity, and mental health. Frontiers in Physiology|Chronobiology. (IF: 4.566)

9. Ludwig, M., Basti, A, **Yalçin, M.,** Schulte, J., H., Relógio, A. (2023). Molecular characterization of the circadian clock in paediatric leukaemia patients: a prospective study protocol. BMC Pediatrics. (IF: 2.567)

10. Malhan, D., **Yalçin, M.,** Schoenrock, B., Blottner, D., Relógio, A. (2023). Skeletal muscle gene expression dysregulation in long-term spaceflights and aging is clock-dependent. npj microgravity. (2-year IF 4.97 (2021))

11. Dose, B., **Yalçin, M.,** Dries, S., Relógio, A. (2023). TimeTeller for timing health: The potential of circadian medicine to improve performance, prevent disease and optimize treatment. Frontiers in Digital Health. (IF: Not applicable, last available IF list updated on 29 June 2022)

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