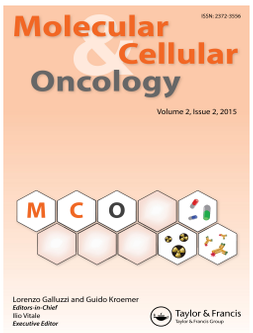

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Loss of Schwann cell's normal rhythmic core clock gene expression and gain of rhythmic expression of oncogenic driver genes in malignant NF1-associated peripheral nerve sheath tumor

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ABSTRACT

Neurofibromatosis type 1 (NF1) is an autosomal dominant tumor syndrome caused by pathogenic variants in the *NF1* gene. Beside tumor formation, patients often have sleep disturbances, suggesting circadian involvement. Previous NF1 studies have implicated MAPK pathway, cAMP-PKA, calcium signaling, and ALK in circadian regulation, and shown disrupted rhythms in murine astrocytes lacking *NF1*. However, whether human Schwann cells show rhythmic gene expression remains unknown, although impaired rhythms may contribute to tumorigenesis. In this study, we analyzed normal human Schwann cells and NF1-derived malignant peripheral nerve sheath tumors (MPNST) for rhythmic gene expression. Cultured cells were synchronized via serum shock, and mRNA levels of core clock and associated genes (e.g. *ARNTL*, *JUN, TGFA, CLOCK, VEGFA, MYC*) were quantified at defined intervals. We observed rhythmic core clock gene expression in normal Schwann cells, demonstrating intrinsic circadian oscillations in peripheral glia. In contrast, MPNST lacked rhythmicity in core clock genes, instead showing de novo rhythmic expression of oncogenes and growth factors like *MYC* and *VEGFA*. Thus, loss of clock gene rhythmicity (desynchronization) and emergence of rhythmic oncogene expression (synchronization) in NF1-associated MPNST further our understanding of peripheral glial physiology and tumorigenesis. These insights suggest that chronotherapeutic strategies may be beneficial for NF1-associated MPNST.

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Circadian; rhythm; Schwann cell; MPNST; NF1; clock gene

Introduction

Neurofibromatosis type 1 (NF1, OMIM 613,113) is an autosomal dominant tumor predisposition syndrome affecting all cells of the body. Clinical diagnostic criteria were updated in 2021[1] and include café-au-lait macules, freckling, neurofibromas, Lisch nodules, optic pathway gliomas, and osseous lesions. Pathogenic germline variants of the highly conserved *NF1* gene (17q11.2) are causative. These variants distribute across the entire coding and noncoding region or can occur as deletions and microdeletions and lead to a reduced expression, stability and activity of the heterodimer neurofibromin in haploinsufficient cells of the body ($NF1^{\pm}$). Neurofibromin is a GTPase-activating protein (GAP) and acts as a tumor suppressor via negatively regulating Ras. The protein is composed of several domains and acts either in an auto-inhibited or open functional state.[2] Pathogenic variants can thereby induce a targeted protein degradation via a destabilization of the wild-type neurofibromin by dominant-negatively affecting dimerization.[3] A failed neurofibromin

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dimerization may explain a general increase in Ras signaling in NF1-associated cells, even if the central GTPase-related domain (GRD) of neurofibromin is not mutated. Failure of the interface between Ras and the GRD explain the high impact of missense mutations such as R1276P on malignant tumor growth in NF1 individuals early in disease development.[4]

NF1 patients face an increased risk for brain tumors, malignant peripheral nerve sheath tumors (MPNST), breast and other cancers.[5] Many other symptoms are associated with NF1. Some of those awaiting sound elucidation such as sleep abnormalities. Although the disturbance of the sleep-wake cycle in NF1 is already known, molecular studies in human cells are very limited. A recent study based on parental reports and demonstrated sleep disturbances in NF1 infants. Those showed difficulties settling into sleep and overall reduced sleep. Reduced night sleep, increased sleep onset latency, arousals, sleep-wake transitions, and parasomnias (sleepwalking, sleep terrors) were also determined in NF1 children using questionnaires.[6–8] In adults with NF1, poor sleep quality was also reported, but discussed to have a multifactorial cause as those adults might have also developed organic NF1-associated problems.[9,10] A higher prevalence of obstructive sleep apnea as well as increased fatigue was also reported in NF1.[11,12] Since sleep abnormalities affect cognition, school performance and psychological status, an essential role of this yet underestimated NF1 symptom is to emphasize. Within animal models, some sleeping abnormalities have been recapitulated so far. Thus, NF1 pig models show some sleeping abnormalities.[13] In 2019, a NF1 mouse model reported higher fragmentation of sleep. In detail, increased non-REM sleep-to-wake and wake-to-non-REM transitions, disturbed maintenance, and a higher percentage of delta power was described.[14]

Most intensive molecular studies of the circadian clock in NF1 were undertaken in Drosophila melanogaster, initially by the Walker lab at MGH (Massachusetts General Hospital, Center for Genomic Medicine, Boston, USA), including own studies using mutant $NF1^{E4}$ (also provided for our work by James A. Walker, MGH) and being recently presented and reviewed by Durkin and coauthors.[15–17] The work by Durkin finally demonstrated the role of neurofibromin for sleep disruptions already in early development, as well as for the regulation of sleep duration and continuity across developmental periods as well as action via Alk-dependent pathways in neurons. Earlier studies unraveled the role of the *NF1* gene on circadian rhythms in locomotor activity via the Ras/mitogen-activated protein kinase (MAPK) pathway and altered circadian oscillation of phospho-MAPK as well as secreted output from clock cells.[16] The C-terminal domain of neurofibromin was also shown to regulate wakefulness in the Mushroom body of Drosophila via reducing cAMP-dependent protein kinase A (PKA) levels and subsequent regulation of calcium levels, although the downstream pathways seemed to depend on cell types involved.[18] Bai and coworkers elucidated that the *NF1* gene has a conserved role to regulate and decrease calcium in diuretic hormone 44 circadian neurons of Drosophila and of mouse astrocytes, which generally show a 24-h rhythm.[19] They demonstrated an interaction of neurofibromin and pigment-dispersing factor (PDF) of central clock cells to generate behavioral rhythms in Drosophila and postulated that neurofibromin affects the outputs of the clock within PDF-expressing cells. Their finding that astrocytes, owing a crucial role in circadian rhythm, demonstrate a disturbed rhythm when depleted of *NF1* is the first experiment to uncover this specific role of *NF1* in mammalian glial cells. As they seem to be important for maintenance of a circadian rhythm in mammals, these recent findings support our hypothesis that also Schwann cells, the peripheral glial cells, might have a disturbed circadian rhythm in NF1.[20]

To summarize from previous animal studies, MAPK and cAMP pathways are involved in mediating the circadian rhythm generating system, as well as its input and output pathways. In human, pathogenic variants of neurofibromin might affect the sleep-wake cycle at least via these two pathways, but if other important domains of the protein are involved is not investigated. Recently, in Drosophila leucine-zipper-like transcription regulator 1 (LZTR1), which also negatively regulates Ras, was identified as a regulator of sleep as well.[21] This underlines the importance of the GRD of *NF1*, similarly. Nevertheless, many other domains of neurofibromin are crucial for highly conserved cellular processes[22] and their influence on rhythmic gene regulation has not yet been ruled out for those.

Circadian rhythms are present in most forms of life.[23,24] The central pacemaker of the human internal circadian clock is the suprachiasmatic nucleus (SCN) of the ventral hypothalamus, which controls physiological rhythms and their synchronization with environmental cycles via zeitgebers (time cues), such as light, food intake, or body temperature. Core clock genes (CCG) drive and maintain circadian rhythms via a transcriptional-translational feedback loop. The positive limb consists of circadian locomotor output

cycles kaput (*CLOCK*) and brain and muscle arnt-like protein (*BMAL1* or *ARNTL*), which form a heterodimer that activates the transcription of target genes, including the negative regulators period (*PER*) and cryptochrome (*CRY*). The PER and CRY proteins form complexes that inhibit the activity of the CLOCK:BMAL1 complex, thereby establishing a ~ 24-h oscillation. CCGs regulate important functions such as cellular metabolism and respiration, the immune system, DNA repair, cell cycle, apoptosis, and chromatin remodeling as well as they control clock-control genes.[25–28] Clock genes are central mediators of the bidirectional communication between the circadian and peripheral physiological processes. The expression and activity of these genes regulate daily rhythms in multiple systems and, in turn, can be modulated by physiological feedback signals. However, the role of these genes is not limited to control the internal clock. Clock genes are also involved in generating phenotypes that are independent of circadian clock function, thus illustrating their pleiotropic effects.[29]

Impaired circadian rhythms and pathogenic variants of clock genes have been shown to drive tumorigenesis and tumor progression in various entities and to contribute to establish cancer hallmarks.[30] Furthermore, variants of CCG are associated with several cancers[31] and malignancies.[32] Activating pathogenic variants of *Ras* are related to an altered circadian rhythm.[33–35] Moreover, an essential component of the transcription-translation feedback loop, the CLOCK:BMAL1 heterodimer, binds to the E-box DNA sequence (CANNTG). This sequence is also recognized by the proto-oncogene myelocytomatose (MYC), which binds as a heterodimer with MYC-associated factor X (MAX), highlighting a shared regulatory motif between the circadian clock and oncogenic pathways.[36] In NF1, neurofibroma is a hallmark of disease arising from peripheral Schwann cells. Development of benign nerve sheath tumors that acquire a second *NF1* hit ($NF1^{-/-}$) depend on different molecular parameters such as the haploinsufficient microenvironment ($NF^{\pm}$), the immune system and other modifying factors, e.g. mismatch repair.[37] For malignant change into MPNST, $NF1^{-/-}$ Schwann cells are supposed to underlie further mutations of cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*), SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 2 (*SMARCA2*), phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*), tumor protein 53 (*TP53*), and polycomb repressive complex 2 (*PRC2*), genetic instability, and/or amplifications of hepatocyte growth factor receptor (*HGF/MET*), MYC, double-strand-break repair protein *RAD21*, cluster of differentiation 117 (*CD117/KIT*), platelet-derived growth factor receptor alpha (*PDGFRA*), epidermal growth factor receptor (*EGFR*) and hepatocyte growth factor (*HGF*), as well upregulation of bromodomain-containing 4 (*BRD4*).

In addition, Melatonin is a neurohormone primarily secreted by the pineal gland during the night, and its rhythmic release is tightly regulated by the central circadian clock located in the SCN of the hypothalamus. Acting as a key output signal of the circadian system, melatonin conveys information about environmental light-dark cycles to peripheral tissues, thereby synchronizing physiological processes to the external day-night rhythm.

We hypothesize that the disruption of rhythmic gene expression is important for tumor growth and malignant change in NF1 as shown for several types of neoplasia. Studies on rhythmic gene expression in NF1-associated tumors and MPNST ($NF1^{-/-}$) do not exist so far. The role of melatonin in the desynchronization of NF-1-associated tumors is also still unclear. Additionally, it is unknown if non-NF1, normal ($NF1^{+/+}$) Schwann cells or NF1-associated non-tumor Schwann cells ($NF1^{\pm}$) show a rhythmic gene expression as described for astrocytes. As malignant progression of peripheral nerve sheath tumors is still a challenge in NF1 patient management, our data might open new avenues for the role of desynchronization in NF1-associated tumors, as well as associated therapy development including chronotherapy.

Methods

Cell lines

Human T265, S462, and NSF1 cell lines of malignant peripheral nerve sheath tumors (MPNSTs) being associated with neurofibromatosis type 1, and BRGN control cells used in this study were previously described.[38–43] T462 and T265 cells were initially provided by Victor Mautner (University of Hamburg, Germany). MPNST were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, 2 mM GlutaMAX™ and 2 mM sodium pyruvate (all purchased from Gibco, Thermo Fisher Scientific, Waltham, USA). The primary

human Schwann cells (HSC) were obtained by ScienCell (#1700, Carlsbad, CA, USA). HSC were cultured in Schwann Cell Medium (SCM, ScienCell) adding 1% Schwann cell growth supplement (SCGS), 5% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. All cell lines were incubated with a humidified atmosphere of 5% CO_2 at 37°C.

mRNA isolation

For each cell line, $2.5x10^4$ cells per well were seeded in 6 well plates (TPP, Trasadingen, Switzerland) in 3 mL complete DMEM medium. After 24 h FBS-free medium was added, and cells were incubated for another 24 h. Then, a serum shock was induced with 10% FBS-containing medium. After that the cells were lysed at six-hour intervals starting at zero and ending at 48 h (Figure 1). For sample collection, cells were washed two times with 1 mL of 4°C cold Phosphate Buffered Saline (PBS, Gibco, Thermo Fisher Scientific) and harvested using 300 µL Lysis Buffer (LBP, MACHERY-NAGEL, Düren, Germany) per well. RNA was isolated with NucleoSpin RNA plus (MACHERY-NAGEL) according to the manufacturer's instructions.

cDNA library preparation and NGS analysis

10 ng mRNA per sample was transcribed into cDNA with AmpliSeq for Illumina Library Prep (Illumina, San Diego, USA) following manufacturer`s protocol. Transcript level of the following genes were analyzed using an Illumina custom panel: Jun proto-oncogene (*JUN*), Transforming Growth Factor Alpha (*TGFA*), Period Circadian Regulator 2 (*PER2*), Prokineticin 2 (PROK2), Clock Circadian Regulator (*CLOCK*), Vascular Endothelial Growth Factor A (VEGFA), MYC proto-oncogene (*MYC*), Mitogen-Activated Protein Kinase Kinase Kinase 8 (MAP3K8), cAMP Responsive Element Modulator (*CREM*), aryl hydrocarbon receptor nuclear translocator like (*ARNTL* or *BMAL1*), Cryptochrome Circadian Regulator 2 (*CRY2*), Cyclin D1 (*CCND1*), *TIMELESS*, Cryptochrome Circadian Regulator 1 (*CRY1*), Period Circadian Regulator 1 (*PER1*), nuclear receptor subfamily 1 group D member 1 (*NR1D1*), D-Box Binding PAR BZIP Transcription Factor (*DBP*), Casein Kinase 1 Epsilon (*CSNK1E*) and for reference Hypoxanthine Phosphoribosyl transferase 1 (*HPRT1)* (Supplementary Table S1). Therefore, library preparation was performed using the AmpliSeq for Illumina Custom Panels procedure (Illumina, San Diego, USA). After library preparation, the samples were analyzed via Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA) for pre-sequencing quantification and library quality control. The Illumina MiSeq instrument (Illumina, San Diego, USA) was used to quantify transcript levels. Therefore, quality-controlled libraries were diluted, equimolar pooled and again diluted to 20 pM according to the MiSeq System Denature and Dilute Libraries Guide performing the Standard Normalization Method (Illumina, San Diego, USA). Finally, the denatured and diluted library pool was loaded onto a MiSeq Reagent Kit v3 cartridge with 13 pM and 1% phiX. The sample sheet, the flow cell, the PR2 bottle, the waste bottle, as well as the reagent cartridge were prepared analogously to the MiSeq System Guide (Illumina, San Diego, USA). For sequencing, a paired end run of 151 cycles per read with FASTQ Only was chosen. Resulting FASTQ files were analyzed with CLC Genomics Workbench 22 (QIAGEN, Hilden,

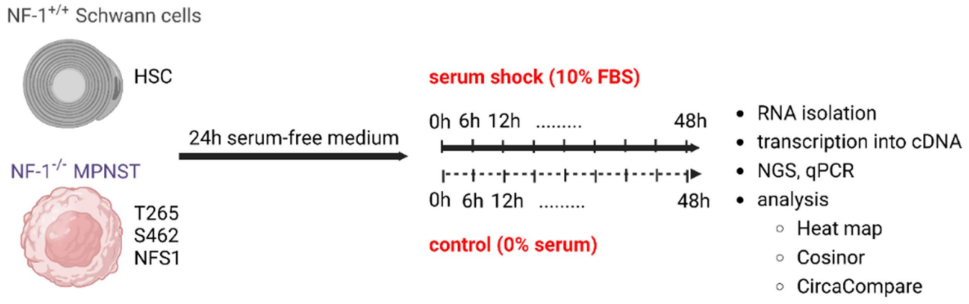

Figure 1. Experimental workflow. Legend: Created with BioRender.com (agreement number: QY25NKONZ9).

Germany). As no transcripts were detected for *PROK2* and *JUN* in all samples, we did not further include them into evaluation.

Quantitative real time PCR

2 µg RNA was transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) with oligo(dT)18-primers according manufacturer's instructions. The cDNA concentration was set at 20 ng/µl and reverse transcription was done in the PEQLAB primus 25 advanced thermocycler (VWR, Avantor, Radnor, USA).

Quantitative determination of Melatonin receptors (MT) was carried out by quantitative real-time polymerase chain reaction (qPCR). The two-step PCR was performed with 10 µl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific, Waltham, USA), 500 nM each of the corresponding forward and reverse primer (Invitrogen, Thermo Fisher Scientific, Waltham, USA,) (Supplementary Table S2) and 1 µl cDNA (20 ng/µl) in a total volume of 20 µl using the Rotorgene Q (Qiagen, Hilden, Germany). The corresponding mRNA values for Glycerinaldehyd-3-phosphat-dehydrogenase (*GAPDH*) were used as control.

Proliferation assays

$5x10^3$ cells per well of MPNST cells and HSC were seeded in quintuplicates in 96-well plates (TPP, Trasadingen, Switzerland). Cell growth was measured using the Cell Proliferation Kit II (Roche, Basel, Switzerland) following the instructions described by the manufacturer. The experiments were carried out for each cell line in DMEM (Gibco, Thermo Fisher Scientific) without serum and supplemented with 10% FBS.

To test viability in response to Melatonin treatment, triplicates of $1x10^4$ MPNST cells (T265, S462 and NSF1) and control fibroblasts (BRGN) were seeded in 100 µL media in a 96 well format and incubated for 24 hours prior to Melatonin (Merck, Darmstadt, Germany) treatment. Then, Melatonin was added, and cells incubated for additional 24 h (physiological presence is normally 12 h during night). For drug treatment, PBS (pH 7.4) was used to dilute Melatonin (0; 0.0025; 0.005; 0.01; 0.1; 1.0 and 10 µM final concentration with 100 µL media per well). After Melatonin treatment, both MPNST and BRGN cells were incubated with 20 µL of CellTiter-Blue® reagent (Promega, Madison, USA) for 1 h and fluorescence (560 nm excitation/590 nm emission) was recorded, normalized and evaluated as previously described.[43]

Immunofluorescence

$5x10^5$ HSC were seeded in 3 mL complete DMEM/F12 per well on poly-L-lysin pre-coated cover slips (2-well Tissue Culture Chambers, Sarstedt, Nümbrecht, Germany) and incubated for 24 h at 37°C and 5% CO_2 during humidified atmosphere. Then, cells were washed with 1.5 mL per well of Hanks' Balanced Salt Solution (HBSS with Ca^{2+} and Mg^{2+}, Thermo Fisher Scientific) containing 3 mmol/L $MgCl_2$. Subsequently, cells were washed two times with HBSS and fixed with cold methanol of −20°C (Merck, Sigma-Aldrich, Darmstadt, Germany) on ice for 10 min. The cells were incubated with 1 ml per well blocking buffer containing PBS, 5% normal goat serum (Cell Signaling Technology, Danvers, USA) and 0.3% Triton X-100 (Carl Roth, Karlsruhe, Germany) for 1 h. Then, S100 mouse monoclonal primary antibody (Cell Signaling Technology, Danvers, USA) diluted 1:1000 in 900 µL per well PBS with 1% bovine serum albumin (Carl Roth) and 0.3% Triton X-100 (antibody diluting solution), was added to the cells for 16–18 h at 4°C. The next day, cells were washed three times with 1 mL PBS followed by incubation with secondary antibody (1:1000, Anti-Mouse IgG (H+L), F(ab')2 Fragment Alexa Fluor 488 Conjugate, Cell Signaling Technology) diluted in antibody diluting solution for 90 min at room temperature in the dark. After washing twice with PBS, the chamber was removed, and the slide rinsed again with PBS. Then, the slides were covered with mounting medium containing DAPI (ImmunoSelect Antifading Mounting Medium DAPI, Dianova, Hamburg, Germany) and dried for 24 hours. The imaging was done with Keyence BZ-800E microscope (Keyence, Neu-Isenburg, Germany).

Data analysis, statistics, and bioinformatic database analysis

NGS data analysis and heat map creation were done using GraphPad Prism software (GraphPad Software, Boston, USA) version 9.5.0. Tests for the presence of a circadian rhythm were performed by Cosinor analysis based on established methods (Refinetti, R.; Circadian Physiology, 3rd Eds.) as described before[44] and using a freely available online tool (https://cosinor.online/app/cosinor.php). In addition, numerical and graphical analysis was done using the CosinorPy Python package.[45] To determine the most suitable period length, a "grid-search" approach is employed, where the parameter T is systematically varied between 12 h and 48 h (based on known biological rhythms) in 1000 equidistant steps, due to computing capacities and time. For each period length, CosinorPy calculates both the p-value and the log-likelihood. For the analysis and graphical representation, the python libraries NumPy,[46] pandas version v2.0.3,[47] matplotlib[48] and SciPy 1.0[49] are utilized. The standard deviation was calculated by fitting a second-degree polynomial to the maximum of each log-likelihood assuming that T is Gaussian distributed and defining a threshold-value of it (maximum_log-likelihood − 0.5). This approach seems to overestimate the standard deviation. The combination of graphical representations for the log-likelihood and p-values as a function of T enables the identification of the most probable period length for each gene.

Further, three types of gene expression were defined, and the results were compared in both serum starvation and serum shock. A rhythmic gene expression was defined as a significant correlation in CosinorPy comparing the expression under serum shock and serum starvation. Expression designed to be inducible when gene expression was triggered by the serum shock compared to serum starvation. Spontaneous gene expression included both induced, rhythmic and completely independent gene expressions.

For analysis of viability under melatonin treatment, we applied statistical testing and graphical representation as previously described in detail.[43]

To identify recently provided data for rhythmic gene expression Schwann cells, we performed a bioinformatic search of the following databases: CGDB (Circadian Gene DataBase), CircaDB (Circadian Expression Profiles Database), and GEO (Gene Expression Omnibus).

Results

Rhythmic gene expression pattern in normal human Schwann cells

Rhythmic RNA expression of clock genes (*ARNTL, CLOCK, CRY1, CRY2, PER1, PER2, TIMELESS*), proto-oncogenes (*MAP3K8, CCND1, MYC*), transcription factors or repressors (*CREM, DBP, NR1D1*), growth factors (*TGFA, VEGFA*), and important kinases such as *CSNK1E* was analyzed. Normal human Schwann cells were shown to strongly express protein S100beta and to exhibit a very low proliferation both under 10% FBS medium supplementation and under starvation (Supplementary Figure S1). The heat map of transcript levels at different time-points was markedly different compared to MPNST cells (Figure 2).

Resolution to single genes demonstrated a rhythmic gene expression with a period length between 15.7 h and 41.0 h of core clock genes *ARNTL/BMAL1, CLOCK, CRY1*, and *CRY2* as well as genes *CSNK1E* and *NR1D1* (Table 1, Supplementary Figure S3) whereas *PER* and *TIMELESS* were not rhythmic. Figure 3 summarizes side by side the rhythmic expression patterns for normal Schwann cells (A) and differences I rhythmicity that occurred in the cell types.

All genes showed both a spontaneous and rhythmic gene expression induced by a serum shock in most of them. The log-likelihood values of *CLOCK* RNA levels were low for shorter period lengths, but they were significantly increased starting from approximately 25 h until reaching a maximum at $T = 41$ h. p-values for *CLOCK* experienced an initial short drop and then reached a peak before declining again and falling below the significance level at just under 35 h (p-value of 0.018 at the point of maximum log-likelihood). The log-likelihood of *CRY1* expression exhibited a swift rise to its maximum at $T = 15.8$ h, even for shorter period durations. Thereafter, the values decreased strongly and did not reach a significant maximum despite a minor increase. P-value drops to its minimum, resulting in an undercut of the significance level when the log-likelihood reached its maximum.

Periods mimicking a circadian rhythm were assumed for core clock genes *ARNTL/BMAL1* and *CRY2* whereas other rhythmic expression patterns did not match a circadian period at all. The log-likelihood for *ARNTL* transcript levels peaks at a period length of 21.5 h, indicating the most probable periodicity.

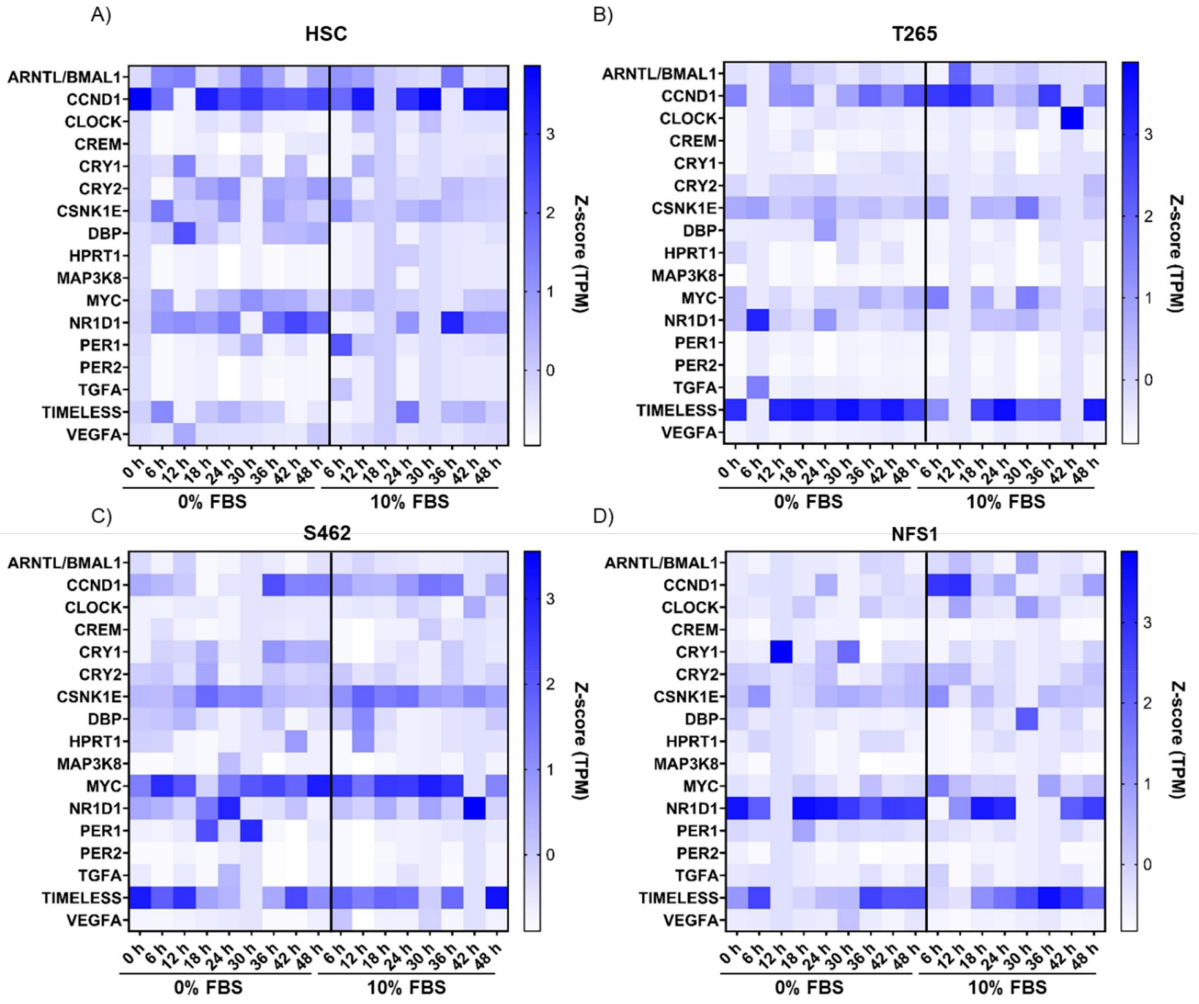

Figure 2. Heatmap of NGS RNA analysis. Legend: Transcript levels of HSC (A) and MPNST cell lines, T265 (B), S462 (C) and NFS1 (D), were analyzed using a custom gene panel of 19 genes. As no transcript level of *JUN* and *PROK2* were detected, they are omitted. Subsequently, Z-score transformed transcripts per million reads (TPM) are depicted against time.

Table 1. Expressions of genes that were rhythmic after a 10% FBS serum shock in normal human Schwann cells.

Gene	Inducible r.	Spontaneous r.	Period length [h]	p-value
ARNTL/ BMAL1	+	+	21.5 ± 0.3	2x10 − 5
CLOCK	+	+	41.0 ± 5.2	.018
CRY1	–	+	15.8 ± 0.6	.042
CRY2	+	+	26.8 ± 2.5	.041
CSNK1E	+	+	15.7 ± 0.4	.002
NR1D1	–	+	28.8 ± 2.3	.031

Legend:All genes also demonstrated a spontaneous rhythmicity (r.) of expression. CRY1 and CSNK1E did not show an inducible rhythmic activity.

The log-likelihood decreased significantly for both shorter and longer periods. When plotting the calculated p-value as a function of the period (significance level of $\alpha = 0.05$), p-value of *ARNTL* declined sharply but reached a plateau of $T = 20$ h onwards with minimal values that persisted until approximately $T = 23$ h before sharply increasing again. The log-likelihood curve of *CRY2* transcription levels showed a descending trend until reaching its minimum, followed by a distinct rise to the maximum at $T = 26.8$ h. Similarly, the p-value initially reaches its maximum, followed by a steep drop to the minimum, and then undergoes a subsequent increase. Once again, the significance level is undercut at the time of the maximum log-likelihood value for *CRY2*. An overview of all gene rhythms found is shown in Supplementary Table S1.

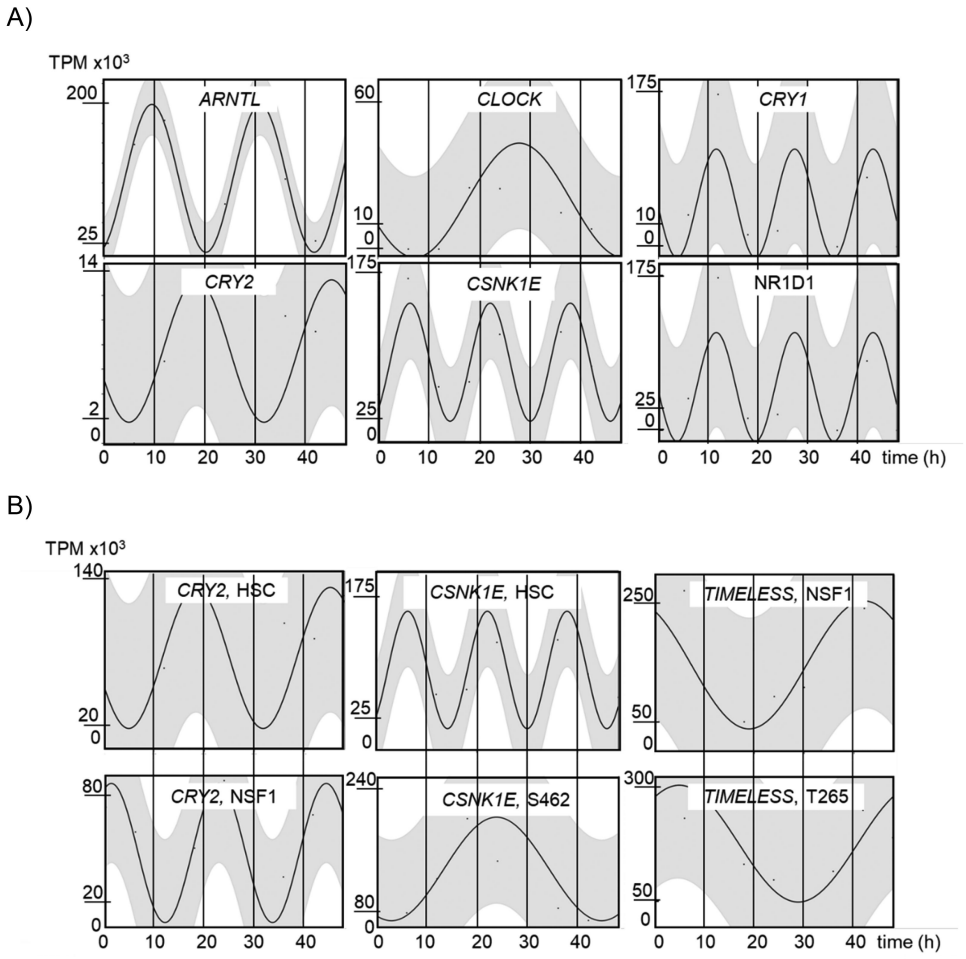

Figure 3. Graphical representation of the results generated by CosinorPy for periodic gene expression. Legend: Periodic gene expression patterns in normal human Schwann cells (A) and, when being different in rhythmicity, in comparison to MPNST cells (B) are shown. Phase-shifted sine-shaped functions with specific periods fitted to the measured TPM data. Threshold of significance is visually highlighted in light grey. All rhythmic gene expression profiles are shown in the supplemental material.

To elucidate the value of rhythmic gene expression in normal Schwann cells, we searched databases storing that information. We only detected information on «tibial nerve» which was retrieved from the GTExPortal (https://gtexportal.org/home/tissue/Nerve_Tibial). These analyses from bulk tissue RNAseq included 619 samples of human individuals of different ages. A specification to a certain cell type such as the Schwann was not deposited.

Altered rhythmic gene expression pattern in NF1-associated MPNST

To demonstrate that serum starvation is an appropriate (negative) control for our experiments, we performed proliferation assays under 0% and 10% FBS supplementation. All three MPNST cell lines demonstrated a very low proliferation under starvation and increased proliferation under normal media supplementation. In detail, under serum starvation, the metabolic activity of MPNST cells were not changing over the time indicating growth arrest. After adding 10% serum, cells showed increased metabolic activity. MPNST cell lines T265 and S462 showed a higher proliferation compared to NFS1 (Supplementary Figure S2).

Table 2. Expressions of genes that were rhythmic after a 10% FBS serum shock in three different MPNST cell lines.

Cell line	Gene	Period length [h]	p-value
T265	*PER1*	30.4 ± 2.0	.004
	DBP	36.1 ± 4.6	.037
	VEGFA	22.7 ± 1.2	.048
S462	*TIMELESS*	48.0 ± 5.4	.027
	MYC	20.6 ± 1.1	.013
	CREM	34.5 ± 2.5	.015
	CSNK1E	41.7 ± 6.0	.025
	CCND1	48.0 ± 4.9	.037
NFS1	*CRY2*	21.8 ± 1.0	.007
	TIMELESS	46.0 ± 5.5	.034

Analysis of clock gene transcript levels of MPNST showed both differences comparing serum-free cell cultures (0% FBS) to serum-shocked (10% FBS) cultures and comparing normal human Schwann cells to MPNST cells (Table 2).

Figure 3 summarizes side by side the rhythmic expression patterns when different for the cell types investigated. Protein kinase gene *CSNK1E* demonstrated a highly increased period length (from 15.7 h in HSC up to 41.7 h) in S462. In contrast, several genes that did not demonstrate a rhythmic gene expression in normal Schwann cells gained a rhythmic expression and displayed mostly prolonged (longer than 24 h) period lengths: clock output genes *PER1* and *TIMELESS*, proto-oncogenes *CCND1* and *MYC*, transcription factors *CREM* and *DBP*, and growth factor *VEGFA*. *MYC* and *CRY2* were the only genes with a period length shorter than 24 h in MPNST. Transcripts of *CREM* and *MAP3K8* were only detectable in S462 cells. Levels of *PER2* were very low in all cells.

To summarize, rhythmic gene expression of core clock genes is lost in NF1-associated MPNST. Genes that encode clock output genes, oncogenes, growth and transcription factors developed a rhythmic expression in MPNST, mainly exhibiting a long period length.

Melatonin reduces proliferation in NSF1 MPNST cells

As MPNST cells displayed a loss of rhythmic core clock gene expression and a disturbed rhythm of those genes that support tumor progression such as *MYC*, we tested the effect of melatonin at different concentrations.

Only NSF1 cells demonstrated a significant response to Melatonin treatment ($p \leq .05$). For other MPNST cells, there was no statistically significant difference compared to control BRGN cells. Nevertheless, an increase of viability at a concentration of Melatonin between c = 0.01 and $0.1x10^{-6}$ M was seen not only for NSF1 but for the other two cell lines (Figure 4).

We additionally aimed to detect Melatonin receptor expression to improve understanding of response failures. We therefore performed quantitative PCR to detect transcription of melatonin receptor 1 and 2 (*MT1*, *MT2*) in normal Schwann cells as well as in all MPNST lines. Using two sets of primers and repetitive analyses including different cDNA preparations, we did not detect expression of *MT1* and *MT2*, neither in MPNST nor in normal Schwann cells, whereas house-keeping gene expression of GAPDH served a sufficient experimental control (data not shown).

To summarize, only 1/3 MPNST demonstrated a significant response to Melatonin treatment probably underlining the highly differential genomic disturbances that might affect several targets at once in these malignant tumors. Investigated peripheral cells did not seem to express *MT1* and *MT2* indicating a complex influence of Melatonin on peripheral signaling.

Discussion

The human body is running on rhythmic cycles, both in central neurons such as of the SCN, and in peripheral cells. The clock of peripheral organs is underlying similar molecular mechanisms and is based on the TTFL involving known clock genes.[50] A circadian gene expression of different normal peripheral tissues has already been demonstrated for pancreatic cells, fibroblasts, skeletal muscle myotubes, liver, lung, kidney,

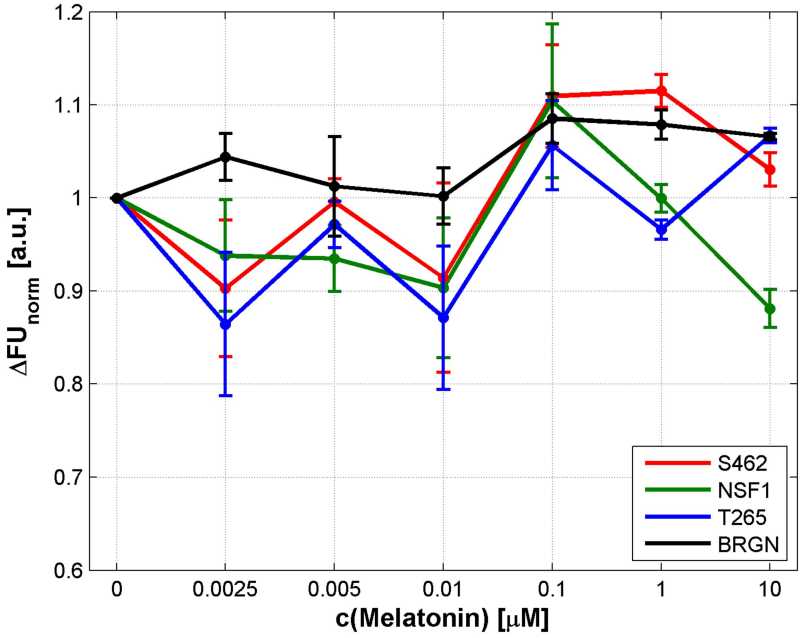

Figure 4. *In-vitro* cell viability under melatonin treatment. Legend: Dose-dependent courses of viability in S462, NSF1, T265, and BRGN cells on Melatonin concentration in a range of 0.0025 up to 10 μM. Mean values of normalized differences in FU according to Eq. (1), and standard errors of the mean (SEM) are presented. We tested two biological replicates with three technical replicates each. Responses of the cell lines are dependent on Melatonin concentration.

spleen, heart, stomach, cornea, thyroid, and adrenal gland, although often in carnivores.[51–54] In mammals, between 2% and 10% of all peripherally expressed genes seem to be rhythmic, whereby liver cells are most often studied with nearly 1000 circadian transcripts demonstrated.[55] Peripheral clocks are self-sustained, autonomous and are entrained by body temperature, signals of SCN, activity cycles, humoral signals such as feeding-related hormones and metabolites, cellular redox state, as well as direct neural signals such as sympathetic and humoral ones, indicating a very strong interplay between energy metabolism and the clock.[55]

For human Schwann cells, the peripheral glial cells, it has not yet been analyzed if genes display a rhythmic expression pattern. The only information that could be retrieved from databases was demonstrating a rhythmic expression of clock genes *ARNTL*, *PER2*, and *CRY2* and some additional genes such as *MYC* in tibial nerves without specifying the cell types. Furthermore, it has not been analyzed if *NF1* deficiency contributes to a rhythmic gene expression in this specific cell type forming the most common tumors in NF1. Our experimental design based on previous experiments that recapitulated the NF1-associated phenotype of abnormal circadian rhythms in Drosophila: using a well-established model, we identified an extreme sleep phenotype in $NF1^{E4}$ mutant flies as we detected a significantly different duration of complete sleep and sleep fragments ($p \leq .01$).[15] Further preliminary experiments using constructs expressing a luciferase gene under the control of the *ARNTL/BMAL* promotor by lentiviral infection to measure rhythmic expression of MPNST T265, S462, and NSF1 demonstrated altered circadian period lengths (Hartmut Halfter, Medical Faculty, University Hospital Münster, personal communication). These pilot tests as well as the recapitulation of an exceptionally disturbed circadian rhythm due to *NF1* deficiency in Drosophila, prompted our further studies in peripheral human Schwann cells.

For the first time, we demonstrate that human peripheral glial cells, Schwann cells, convincingly demonstrate expression rhythmicity of the *ARNTL* and other core clock genes. This is not surprising as the role of the circadian clock for nerve conduction velocity, differentially affecting sensory and motor fibers has been described previously and reviewed by Klymenko and Lutz.[56] Klymenko and Lutz also suggested that Melatonin receptors 1 and 2 (MT1, MT2) may function as "chronosensors" to Melatonin changes, thereby allowing peripheral Schwann cells to sprout and to reassemble myelin as well as to undergo proteolysis, depletion, degradation, and sorting and that clock gene mutations also may influence the

Schwann cell's myelination process in disease.[56] In 1959, Pomerat described a rhythmic contraction of 4 to 18 min of rat Schwann cells which was similar to oligodendroglia.[57] These findings were substantiated by further studies including those that suggested infradian cycles of non-myelinating Schwann cells to regulate hematopoietic stem cell activity.[58] In conclusion of a rat model, Kim *et* al. already suggested that satellite glial of dorsal root ganglia have a rhythmic expression of *ARNTL* and *PER2*[59] being the first hint to a rhythmic gene expression of specialized ganglionic glial cells in rats, the so-called satellite cells that might represent a pre-stage in Schwann cell differentiation.[60,61] In mice, expression of several clock genes (*PER1, PER2, PER3, CRY, CRY2, ARNTL, ARNTL2*) in Schwann cells was described to differ between subtypes of Schwann cells, however not addressing rhythmicity.[62] Now, adding knowledge on a rhythmic gene expression of core clock genes in human Schwann cells fills a gap and facilitates interpretation of altered expression patterns in tumors. Interestingly, the periods show high variability which might be explained by a true individual difference that might be synchronized in an intact organism.

As mentioned before, a mediation of neurofibromin's effects through cAMP signaling and Ras cascade on clock gene expression has been proven in model systems. However, a direct protein-protein interaction has not been ruled out yet. Experimental data that demonstrate an effect of *NF1* deficiency on the rhythmic gene expression in human NF1-derived nonmalignant tumor cells is missing. Nevertheless, we are demonstrating abolishment of the rhythmic gene expression in MPNST and speculate that this might also result from *NF1* loss of function. Interestingly, we did not only detect a loss of core clock gene expression but also a new rhythmic expression of tumor driving genes that were not detectable in normal Schwann cells (Figure 5).

In detail, a rhythmic expression of *MYC* oncoprotein would probably change the regulation of many genes that harbor an E-box, such as p27, thereby changing the regulation of proliferation, cell growth, self-renewal, cell cycle control, and other important cellular processes. It is known that a downregulation of *ARNTL* is associated with increased expression of *MYC* in tumors.[36] There might be two scenarios: On the one hand, it can be speculated that both influence of *ARNTL* and *CLOCK* regulation is lost in favor of *MYC* due to *NF1* mutations in MPNST. On the other hand, enhanced *MYC* expression was also shown to disrupt clock gene oscillation.[63] No rhythmic *MYC*

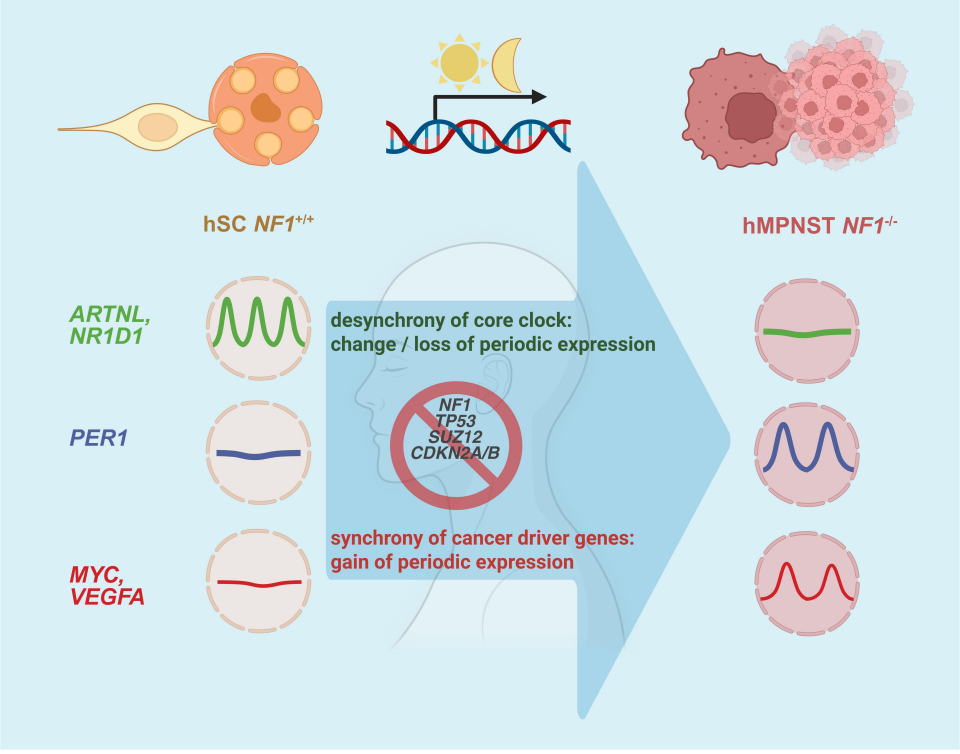

Figure 5. Altered rhythmic gene expression in NF1-associated peripheral nerve sheath tumor cells. Legend: Created with BioRender.com (agreement number IR264V11RF).

expression was detected in MPNSTs with altered rhythmic *PER1* or *CRY2* expression. This suggests, altered *PER1* or *CRY2* expression might be an individual mechanism involved in the progression of MPNST. Since *MYC* is regulated post-transcriptionally via *PER1*, disturbance of clock gene expression due to *NF1* mutations might play a role in the two MPNST cell lines that did not show a novel rhythmic MYC expression.[64]

As we detected loss of rhythmic core clock gene expression in MPNST and gain of rhythmic expression of some tumor driving genes, we tried to elucidate if abnormal rhythmicity can be rebuilt by Melatonin treatment as shown for other tumors, e.g. adenocarcinoma.[65] But we did not detect Melatonin receptors MT1 and MT2 on mRNA level. Still one MPNST responded to relevant dosages. Dose application was according to literature describing serum Melatonin concentrations in normal young adults between 40 and 260 pmol per liter,[66] and a decrease of growth was detected at physiological doses among 100 nM. Since Melatonin is known to have inhibitory effects on various cancers, the response of NSF1 cells to treatment is concordant with literature. The hormone affects the circadian machinery by inhibiting the proteasome and regulating the circadian clock post-translationally, it also represses MYC via *ARNTL, PER*, and *CRY*.[67] Cellular processes induced by Melatonin are complex, and we here propose that another way of action in peripheral Schwann cell and their derived tumors is approached than via receptors MT1 and MT2.[67] The observed distinct response to Melatonin treatment could be caused to at least two factors. Firstly, the genetic heterogeneity of MPNST and an increase in the rhythmic expression of cancer driver genes leads to an uncoupled regulation of different signaling pathways. As previously published, NF1-associated MPNST cell lines show a high genetic diversity, at least shown for S462 and T265.[68] Although the three cell lines all exhibit the typical molecular changes associated with MPNST, the cell lines differ significantly concerning the number of chromosomal rearrangements, type of NF1 mutations, and further unknown genetic features. Additionally, NSF1 is the only cell line that did not show a gain of rhythmic expression of cancer driver genes. Thus, NSF1 cells might exhibit a higher response, because feedback loops of the core clock gene expression may not be as highly disturbed compared to the other MPNST cell lines, which probably suffer from a complete disruption of chronosensitivity.

Finally, we could hypothesize that if altered *NF1* is leading to disturbances of rhythmic gene expression in many cells of the body, taking melatonin could help NF1 patients in principle as the desynchronization of body cells is generally improved.

Conclusion

We demonstrated a rhythmic expression of core clock genes *ARNTL/BMAL1, CLOCK, CRY1*, and *CRY2* in normal human Schwann cells for the first time. Thus, we fill a gap of knowledge and demonstrate that human peripheral glia is also running on autonomous cycles as was suspected previously and shown for other human cell types.

Furthermore, we could prove that Schwann cells' normal rhythmic core clock gene expression is abolished in different NF1-associated MPNST. This might be a result of NF1 gene loss and/or of other genomic alterations in these highly malignant tumors. Desynchrony of rhythmic expression of clock genes and synchrony by gain of rhythmic expression of tumor driver genes such as *MYC* and *VEGF* in NF1-associated MPNST are novel findings that stimulate more detailed studies and chronotherapy approaches.

In conclusion, the lack of reference data and the divergent responses observed in our study emphasize that elucidating the precise molecular pathways and the relevance of MT receptors in human Schwann cells and MPNST requires further investigation.

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Author contributions

CRediT: **Sandra Leisz:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; **Antonio Pelligrino:** Data curation, Formal analysis, Methodology, Writing – original draft; **Saskia Fritzsche:** Investigation, Visualization, Writing – review & editing; **Merle Wiegers:** Software, Visualization, Writing – original draft; **Markus Wösle:** Formal analysis; **Christian Linke:** Data curation, Formal analysis, Visualization; **Swanhild Lohse:** Investigation; **Daniel Tippner:** Writing – review & editing; **Christian Scheller:** Funding acquisition, Resources, Supervision; **Christian Strauss:** Resources, Supervision; **Eva Ehrentreich-Förster:** Resources, Writing – review & editing; **Faramarz Dehghani:** Investigation, Methodology, Resources, Supervision; **Stanislav Sys:** Formal analysis; **Erik Maronde:** Supervision, Validation, Writing – original draft; **Anja Harder:** Conceptualization, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data is provided within the manuscript and/or supplementary information files. Raw data were uploaded to Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/.) on 15th July, 2024, using the Metadata file name: seq_template.xlsx (space subfolder: uploads/immu_halle_qtS3u6Au/NGS MPNST. Accession number: will be provided on acceptance. The analyzed datasets of the current study are also available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Direct material of human individuals was not analyzed. None of the human cell lines required ethics approval. Data on cell lines are cited in the material and methods section.

List of abbreviations

BMAL1/ARNTL:	Brain and muscle Arnt-like protein
BRD4:	Bromodomain-containing 4
cAMP:	Cyclic adenosine monophosphate
CCG:	Core clock genes
CCND1:	Cyclin D1
CD117/KIT:	Cluster of differentiation 117
CDKN2A/B:	Cyclin-dependent kinase inhibitor 2A/B
cDNA:	Complementary DNA
CLOCK:	Circadian locomotor output cycles kaput
CNS:	Central nervous system
CREM:	cAMP Responsive Element Modulator
CRY:	Cryptochrome
CRY1:	Cryptochrome Circadian Regulator 1
CRY2:	Cryptochrome Circadian Regulator 2
CSNK1E:	Casein Kinase 1 Epsilon
CTB:	CellTiter-Blue®
CU:	Colorimetric units

DBP:	D-Box Binding PAR BZIP Transcription Factor
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	Desoxyribonucleic acid
EGFR:	Epidermal growth factor receptor
F12:	Ham's F-12
FBS:	Fetal bovine serum
FU:	Fluorescence units
GAP:	GTPase-activating protein
GRD:	GTPase-related domain
HBSS:	Hanks' Balanced Salt Solution
HGF/MET:	Hepatocyte growth factor receptor
HGF:	Hepatocyte growth factor
HPRT1:	Hypoxanthine Phosphoribosyltransferase 1
HSC:	Human Schwann cells
IgG:	Immunoglobulin G
JUN:	Jun proto-oncogene
Ki:	Kiel
LBP:	Lysis Buffer
LZTR1:	Leucine-zipper-like transcription regulator 1
MAP3K8:	Mitogen-Activated Protein Kinase Kinase Kinase 8
MAPK:	Mitogen-activated protein kinase
MPNST:	Malignant peripheral nerve sheath tumor
MYC:	Myelocytomatose
NF1:	Neurofibromatosis type 1
NGS:	Next Generation Sequencing
NR1D1:	Nuclear receptor subfamily 1 group D member 1
PBS:	Phosphate Buffered Saline
PDF:	Pigment-dispersing factor
PDGFRA:	Platelet-derived growth factor receptor alpha
PER:	Period
PER1:	Period Circadian Regulator 1
PER2:	Period Circadian Regulator 2
PKA:	Protein kinase A
PRC2:	Polycomb repressive complex 2
PROK2:	Prokineticin 2
PTEN:	Phosphatase and tensin homolog deleted
RAD21:	RAD21 cohesin complex component
Ras:	Rat sarcoma
REM:	Rapid eye movement
RNA:	Ribonucleic acid
SCGS:	Schwann cell growth supplement
SCM:	Schwann Cell Medium
SCN:	Suprachiasmatic nucleus
SEM:	Standard errors of the mean
SMARCA2:	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
SWI/SNF:	SWItch/Sucrose Non-Fermentable
TGFA:	Transforming Growth Factor Alpha
TP53:	Tumor protein 53
TPM:	Transcripts per million reads
TTFL:	Transcription/translation feedback loop
VEGFA:	Vascular endothelial growth factor A

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