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Critical cholangiocarcinogenesis control by cryptochrome clock genes

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List of abbreviations: Arb. Units: Arbitrary Units; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatases; HCC: Hepatocarcinoma; CCA: Cholangiocarcinoma; *Cry*: Cryptochrome; *Cry1*$^{-/-}$*Cry2*$^{-/-}$: *Cry1* and *Cry2* double deficiency; CTS: Circadian Timing System; DEN: Diethylnitrosamine; Per: Period; TTFL: Transcription–Translation Feedback Loop; WT: Wild Type; ZT: Zeitgeber Time.

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Abstract

A coordinated network of molecular circadian clocks in individual cells generates 24-hour rhythms in liver metabolism and proliferation. Circadian disruption through chronic jet lag or *Per2* clock gene mutation was shown to accelerate hepatocarcinoma development in mice. Since divergent effects were reported for clock genes *Per* and *Cry* regarding xenobiotic toxicity, we questioned the role of *Cry1* and *Cry2* in liver carcinogenesis.

Male WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (C57Bl/6 background) were chronically exposed to diethylnitrosamine (DEN) at ZT11. Rest-activity and body temperature rhythms were monitored using an implanted radiotransmitter. Serum aspartate and alanine aminotransferases (AST, ALT) were determined on four occasions during the progression stage. After 7 months, serum alkaline phosphatases (ALP) were determined, and livers were sampled for microscopic tumor nodule counting and histopathology.

Five months after initiation of DEN treatment, we found that *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice developed severe liver dysplasia, as evident from the increased AST, ALT and ALP levels, as compared to WT mice. DEN exposure induced primary liver cancers in nearly fivefold as many *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT mice (p= 0.01). Microscopic study revealed no difference in the average number of hepatocarcinomas and a nearly 8-fold increase in the average number of cholangiocarcinomas in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, as compared to WT mice.

The study validated the hypothesis that molecular circadian clock disruption dramatically increased chemically-induced liver carcinogenesis. In addition, the pronounced shift towards cholangiocarcinoma in DEN exposed *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice revealed a critical role of the *Cry* clock genes in bile duct carcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) constitute the vast majority of primary liver cancers, the third cause of cancer-related mortality worldwide. These liver cancers are derived from the respective malignant transformation of hepatocytes and bile duct epithelial cells [1, 2]. Diethylnitrosamine (DEN) is a hepatic carcinogen that usually induces HCC in rodents, which have been considered as a good reflection of poor prognosis human HCC [3]. DEN also induces CCA in mice with bile duct ligation [4, 5]. In this study, we used DEN as a liver cancer initiator and promoter to investigate the role of circadian genes *Cry1* and *Cry2* for liver carcinogenesis in mice.

The mammalian Circadian Timing System (CTS) regulates behavioral and physiological processes over the 24 hours. Endogenous circadian rhythms are mainly synchronized to the regular day-night cycle by light. The liver is one of the most rhythmic organs in the body. Prior studies show that approximately 15% of liver transcriptome and proteome display circadian variations [6, 7]. In addition, a wide range of critical liver metabolic pathways is controlled by the circadian clock, involving amino acid, carbohydrate, lipid, nucleotide, and xenobiotic metabolism [8, 9]. The CTS is coordinated by the suprachiasmatic nucleus (SCN) in the hypothalamus, which acts as a central pacemaker. The SCN generates rhythmic physiology and coordinates molecular circadian clocks in peripheral organs, including the liver [10, 11]. The molecular clock, which resides within almost all cells, consists of at least three interconnected transcription–translation feedback loops (TTFL) that control rhythmic gene expression patterns with a near 24 h period [12]. The core TTFL is driven by activator proteins BMAL1 and CLOCK or NPAS2, which activate the transcription of target genes containing E-box cis-regulatory enhancer sequences, including *Period* (*Per*) *1*, *2*, and *3* genes and *Cryptochrome* (*Cry*) *1* and *2* genes. In turn, PER::CRY protein heterodimers accumulate in

the nucleus and suppress transcription of their own genes by acting on the CLOCK::BMAL1 protein complex, and as such form the main inhibitory loop of the TTFL [11].

Several epidemiologic studies, as well as an international expertise by the World Health Organisation have concluded that "shift-work that involves circadian disruption is probably carcinogenic to humans" [13, 14]. Indeed, circadian disruption promotes tumor initiation and accelerates experimental cancer progression in mice with SCN ablation, experiencing chronic jet lag, or circadian gene mutations, while oppositely CTS reinforcement with meal timing inhibits cancer growth [15-20]. Host circadian disruption was further associated with fast tumor progression and poor survival in cancer patients [21, 22].

Circadian disruption induced by *Per2* mutation, constant light or chronic jet lag exposure has been shown to promote DEN-induced liver carcinogenesis, resulting in increased formation of a majority of HCC and few CCA in rats and mice [23-25]. DEN is usually used for liver cancer initiation (2-4 weeks of DEN exposure) and induces the formation of dysplastic foci with altered hepatocytes. Prolonged DEN administration is used to act as a cancer promoter (week 4-10), and induces irreversible hepatocellular nodules [26, 27]. Recently, it has been shown that *Per2* clock gene acts as a tumor suppressor in the liver of mice exposed during 7 weeks to DEN. *Per2* mutation accelerates hepatocarcinogenesis through uncontrolled proliferation, genomic instability, and tumor promoting inflammation. This mutation increases HCC several fold without any significant effect on CCA development [25].

Cry1 and *Cry2* are involved in the negative loop of the circadian molecular clock. They are robustly expressed and rhythmic in the mouse liver [16, 25]. Thus the combined inactivation of *Cry1* and *Cry2* in mice suppressed rest-activity, core body temperature and corticosterone secretion following constant darkness exposure [28, 29]. The lack of CRY proteins, as in *Cry1/Cry2* double deficient mice, suppressed the rhythmic expressions of *Per1* and *Per2* [30] and other E-Box clock or clock-controlled genes [6, 31]. In addition, *Cry1* and *Cry2* genes

displayed lower RNA messenger expression, phase shift and dampened amplitude during the initiation stage in the liver of WT and *Per2* mutant mice exposed to DEN [25].

In order to further test the clock disruption hypothesis as a liver co-carcinogen, we hypothesized that chronic exposure to DEN would enhance the rate of liver cancers in mice with *Cry1/Cry2* double deficiency as compared to WT mice exposed to the same dose of DEN. We show that *Cry1/Cry2* deficiency increased liver carcinogenesis nearly 5-fold and caused a very pronounced shift in tumor spectrum from HCC to CCA.

Materials and Methods

Mice and Study design

This study was conducted in accordance with the guidelines approved for animal experimental procedures by the French Ethical Committee (decree 87-848).

The effect of *Cry1/Cry2* deficiency for DEN-induced liver cancer was investigated in male C57Bl/6 mice. Eighteen Wild-Type (WT) and 17 $Cry1^{-/-}Cry2^{-/-}$ mice (obtained from $Cry1^{-/-}Cry2^{+/-}$ x $Cry1^{+/-}Cry2^{-/-}$ intercrosses) aged 8 to 16 weeks were used. Mice were kept under a regular 12 hr light/12 hr dark schedule (LD12:12) at ambient temperature (21°C-23°C) for at least 3 weeks before starting DEN administration and throughout experiment, with food and water *ad libitum*. Light intensity at cage level ranged from 240 to 580 lux according to cage location. The lights were on from 4:00 (Zeitgeber time, ZT0) to 16:00 (ZT12).

Sixteen WT and 15 $Cry1^{-/-}Cry2^{-/-}$ mice received a cumulative dose of 406 mg/kg of DEN (Sigma-Aldrich, Saint Quentin Fallavier, France), using a 5-days on and 2-days off schedule over an overall exposure duration of 68 days. Two control mice per genotype received NaCl 0.9%. DEN was administered intraperitoneally (i.p.) at ZT11 (1h before darkness onset).

Treated mice received daily DEN doses of 12 mg/kg from day 1 to 17, i.e. during the carcinogenesis initiation stage. Mice were left DEN-free from day 18 to 28. During the subsequent promotion stage, DEN-treated mice received a dose of 10 mg/kg from day 29 to 33, followed with a 9-days interruption, then re-exposure to 10 mg/kg daily from day 43 to 68. Body weight was recorded daily before each DEN injection and three times a week thereafter. Mortality was checked daily throughout the experiment. Blood was sampled from the retro-orbital sinus on days 142, 164, 186, and 211, i.e. during the progression stage. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined four times during the progression stage in the sera using a Synchron LX20 Clinical System (Beckman Coulter, Villepinte, France). Alkaline phosphatases (ALP) serum levels were measured at the last sampling time point. We considered that day 1 to day 21 corresponded to the initiation stage, day 22 to day 68 to the promotion stage, and day 69 and thereafter to the progression stage. All the mice were euthanized 211 days after DEN exposure onset. Livers, kidneys, and lungs were removed for pathology and histological studies.

Circadian biomarkers

Prior to DEN-treatment, a Physio Tel TA 10 TA-F20 radiotransmitter (Data Sciences, St. Paul, Minnesota) was implanted into the peritoneal cavity under isoflurane anaesthesia in 28 mice, i.e. 14 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ and 14 WT. These telemetric sensors recorded the rest-activity and body temperature rhythms every 10 minutes for 5 days before DEN exposure and for 150 days thereafter.

Histological studies

Immediately after sacrifice, mice were autopsied and macroscopic tumor nodules were counted. Liver, lungs, and kidneys were collected from each mouse for serial histological

slides. All tissues were fixed with 4% formaldehyde for 24 hours, dehydrated, and embedded in paraffin. Then, three 4 µm-thick sections were obtained 1 mm apart in each liver and stained with hematoxylin-eosin-saffron. Slides were examined blindly for the presence and the type of microscopic tumor nodules by a senior pathologist (C.G).

Data analyses

Quantitative data were expressed as mean ± standard error of the mean (SEM). Intergroup differences were compared using multiple-way analyses of variance (ANOVA), Chi-square, T-student and log-rank tests, according to data characteristics. Body temperature and activity data were obtained using Dataquest software. The statistical significance of sinusoidal rhythmicity was documented by cosinor analysis. This method estimates the rhythm characteristics: mesor (24-h mean), amplitude (half of the difference between maximum and minimum value of fitted cosine function) and acrophase (time of maximum in fitted cosine function, with light onset as phase reference). A rhythm period $\tau = 24$ hours was considered *a priori*. A P-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistical analysis software version 18 (Statistical Package for Social Sciences, Chicago, IL).

Results

Systemic toxicity of DEN in WT and *Cry1$^{-/-}$Cry2$^{-/-}$* mice

Mean body weight of both control WT and *Cry1$^{-/-}$Cry2$^{-/-}$* increased slowly over the initial 60 study days, corresponding to the administration of control solution (by 1.2 and 6.5%, respectively). Mean body weight subsequently increased by 9% in WT and 23% in *Cry1$^{-/-}$Cry2$^{-/-}$* mice on day 180, as compared to day 1.

After the start of DEN treatment, a marked body weight loss was observed both in WT and in *Cry1$^{-/-}$Cry2$^{-/-}$* mice, during the carcinogenesis initiation stage. This led us to reduce the daily

DEN dose from 12 to 10 mg/kg. Overall, body weight loss was largest in WT mice as compared to *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (ANOVA, $p< 0.001$). Maximum mean body weight loss was 13.9 ± 1.8 % in WT mice, while it was 7.6 ± 1.5 % in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Figure 1A). Complete body weight recovery was nearly twice as fast in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ as compared to WT mice (42 *vs* 92 days).

No control mouse died. A single mouse in each genotype group receiving DEN died from acute toxicity over the initial two-months' span. DEN-induced mortality was encountered in 2/16 WT mice (13%) as compared to 6/15 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (40%) over the 7-months following DEN exposure onset (Log-rank test, $p= 0.1$) (Figure 1B). Both of the dead WT mice displayed histological evidence of liver inflammation and dysplasia, in association with severe body weight loss on days 41 (-40%) and 161 (-15%) respectively. In contrast, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice displayed (a) severe liver inflammation and dysplasia including apoptotic and necrotic hepatocytes, in association with body weight loss (two mice dead on days 33 and 131), (b) precancerous liver dysplasia (two mice dead on days 189 and 194), and (c) liver tumor nodules (two mice dead on days 204 and 205). Therefore, one death was observed in each group during the promotion stage (day 41 for a WT mouse and day 33 for a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse), while one WT mice and five *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice died during the progression stage. The tumor nodule counts for these latter two *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice dead on days 204 and 205 were combined with those found in the animals euthanized on day 211.

Serum ALT, AST, and ALP levels were only measured during the progression stage. Serum ALT activity was significantly increased in mice of both genotypes on days 186 and 211 only, with levels that were nearly doubled in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT animals ($p< 0.05$) (Figure 1C). From days 142 to 211, AST serum activity was increased in DEN-treated WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, as compared to untreated control animals. AST activity was ~1.6-fold as high on days 142 and 164, and twice as high on days 186 and 211 for *Cry1*$^{-/-}$*Cry2*$^{-/-}$

treated mice as compared to treated-WT ($p< 0.01$ to $p< 0.05$) (Figure 1D). ALP levels remained unaltered in DEN treated WT mice, while they were doubled in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to untreated control animals. Indeed, on DEN exposure, serum ALP concentration increased three-fold in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT mice on day 211 ($p< 0.01$) (Figure 1E).

Baseline rest-activity and body temperature patterns in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice before DEN exposure and throughout the liver carcinogenesis process

Rest-activity and body temperature circadian biomarkers were recorded by telemetry, starting 5 days before DEN exposure onset and continuing until 150 days after it. Prior to carcinogen administration, regular 24-h changes were found for rest-activity and body temperature in WT mice. The 24-h patterns in both variables were severely altered in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Figure 2A). As a result, cosinor analysis validated statistically significant rhythms, with different characteristics according to genotype. Thus, a phase advance was found in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice by 3 h and 10 min for rest-activity (ZT15:40 *vs* ZT18:50; $p<0.001$) and by 1 h and 50 min for body temperature (ZT16:20 *vs* ZT18:10; $p= 0.025$) as compared to WT (Figure 2B). The circadian amplitude was nearly reduced by half for both rest-activity (Hoteling t-test for amplitude comparisons, 1.05 ± 0.27 *vs* 2.09 ±0.22 arb. units, $p< 0.001$), and body temperature (0.53 ± 0.07 *vs* 0.96 ± 0.02°C, $p< 0.001$) in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animals as compared to WT mice (Figure 2C).

In *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, DEN administration supressed the circadian rhythm in rest-activity for 6/14 mice (40%) and that in body temperature for 4/14 animals (28%) during the initiation and/or promotion and progression stages. In contrast, no circadian rhythm loss was observed for both variables in WT mice. Interestingly, the rest-activity and/or temperature circadian

rhythms were completely suppressed during the initiation/promotion stages for four of the six *Cry1*$^{-/-}$ *Cry2*-$^{/-}$ mice, which subsequently died before study completion.

During the initiation stage, DEN exposure further advanced the rest-activity circadian phase by 2 hours in WT ($p < 0.001$), without any significant alteration in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to baseline values before DEN exposure. In contrast, the temperature circadian phase was advanced by ~1 h in WT and ~1 h and 30 min in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (paired T-test, $p < 0.001$) (Figure 2B). In WT animals, DEN halved the circadian amplitude for rest-activity and damped it by 20% for body temperature as compared to their respective baseline values (Hoteling t-test for amplitude comparisons, $p < 0.001$ and $p = 0.03$ respectively). The circadian amplitude for rest-activity rhythm was halved in DEN treated *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($p = 0.003$) while it was 1.6-fold lower for body temperature rhythm as compared to baseline values (Figure 2C).

A nearly 1-h phase advance was statistically confirmed during the promotion stage for both rest-activity and body temperature rhythms in WT mice ($p = 0.09$ and $p < 0.001$ respectively) as compared to their baseline values before DEN exposure. *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice did not display any significant change for rest-activity phase, while cosinor analysis validated a 2-h phase advance for body temperature (ZT16:20 *vs* ZT14:30, $p = 0.03$) (Figure 2B). During this carcinogenesis stage, DEN dampened the circadian amplitude by 37% and 15% for both rest-activity and body temperature rhythms respectively in WT mice ($p \leq 0.001$). Moreover, this effect was more pronounced in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, and the circadian amplitude was nearly halved for both rhythms ($p=0.01$ for rest-activity, and $p=0.03$ for body temperature) (Figure 2C). After DEN exposure, the circadian acrophases and amplitudes of both rhythms recovered back to baseline values in the mice from both genotypes. Double-plot representations of both body temperature and rest-activity rhythms in individual mice over 130 days showed stable rhythms in WT controls mice and markedly damped, yet persistent rhythms in control *Cry1*$^{-/-}$

Cry2$^{-/-}$ animals (Figure 3). Indeed, the daily intraperitoneal injections of the control solution induced minimal if any alteration in the rest-activity and intraperitoneal temperature patterns in both control WT and both control *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, which matched the corresponding DEN-treated groups (Figure 3, Suppl Tables 1 and 2). DEN exposure transiently disrupted both circadian rhythms during the initiation and the promotion stages in WT mice. Both rhythms subsequently recovered during the progression stage. In contrast, the disruption of both rhythms was complete and sustained until the end of the recording in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice exposed to the same DEN cumulative dose (Figures 3A and B).

DEN induced liver carcinogenesis in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

Macroscopic nodules, together with small and large liver cell dysplasia, inflammatory infiltration and apoptotic hepatocytes, were observed in 17 of 25 remaining mice at 7 months (Figure 4 A and B). Control mice with either genotype exhibited normal liver histology (Figure 4 C and D). However, DEN exposure resulted in many more primary biliary lesions in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ as compared to WT mice (Figure 4 E and F). Both CCA's and HCC's could be found in animals from both genotypes (Figure 4 G and H). DEN also induced lung tubulopapillary tumors in 5 WT and 3 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. A single primary tumor was found in the kidney of one *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse.

Liver tumors were found in 43 % of WT and in all *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($p< 0.001$). The average number of tumors per animal including CCA and HCC was nearly five-fold higher in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT animals (Kruskal-Wallis, $p= 0.01$) (Figure 5A). The number of tumor nodules per liver ranged from 0 to 7 in WT and from 1 to 25 in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Fisher's exact test, $p= 0.003$). In addition, the proportion of mice with more than 2 tumors was ~six-fold higher in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT animals (80% *vs* 14%, Fisher's exact test, $p= 0.01$).

In *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, histopathological analysis revealed ~24 times as many CCA as compared to HCC (73 *vs* 3; $p < 0.001$). However, WT mice did not display any significant difference for liver cancer types (Figure 5B). The average number of CCA per mouse was 7.8 fold as high in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared to WT mice ($p < 0.01$), while no validated difference was detected for HCC (Figure 5B).

Discussion

Here we validated the hypothesis that chronic exposure to DEN enhanced several-fold the rate of liver cancers in mice with a *Cry1/Cry2* double deficiency as compared to WT mice exposed to the same dose of DEN. This was consistent with the findings we earlier reported in *Per2*$^{m/m}$ mice, which harboured another clock gene deficiency [25]. However histopathological examination by a senior liver cancer pathologist revealed that the vast majority of liver cancers were cholangio- rather than hepato-carcinomas in the *Cry1/Cry2* double deficient mice. To our knowledge, this is the first study that reveals a direct role of *Cry1* and *Cry2* genes in CCA. Notably, the deficiency of *Cry1* and *Cry2* caused a nearly 8-fold increase in the average number of CCA induced by the hepatic carcinogen DEN in mice.

Cry1$^{-/-}$*Cry2*$^{-/-}$ mice displayed genetic circadian disruption, as indicated by arrhythmic body temperature, rest-activity, and corticosterone secretion while in constant darkness [28, 29]. However, a 24-h rhythm in rest-activity and body temperature was found here in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice exposed to LD12:12, yet with 2 to 3-h phase advance and halved amplitude. These apparent rhythms in clock-deficient *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice likely originated from direct responses to light, so called masking effect [28].

Cry1/Cry2 double deficient mice were also shown to display enhanced susceptibility to develop spontaneous or gamma radiation-induced HCC as compared to WT animals, without

any reported evidence of CCA formation in their livers [19, 32]. In contrast, we did find both HCC's and CCA's in WT B6D2F1 mice kept in LD12:12 or chronically exposed to jet lag and receiving DEN [24], as well as both in WT and in *Per2*$^{m/m}$ mice on chronic DEN [25]. The current study revealed a low incidence of DEN-induced HCC both in WT and in *Cry1/Cry2* double deficient mice on C57Bl/6 background. Yet the large and highly statistically significant difference in CCA formation most likely resulted from altered DEN metabolism and toxicodynamics in the *Cry1/Cry2* double deficient mice. The differences in DEN metabolism and toxicodynamics between *Cry1/Cr*y*2* double deficient and *Per2*$^{m/m}$ mice thus appeared to be crucial for the cancer type outcomes.

The role of *Cry1/Cry2* double deficiency for liver carcinogenesis was here investigated in mice on a C57Bl/6 background, with altered melatonin secretion. Gas chromatography (GC)-mass spectrometry (MS) studies have demonstrated melatonin in the pineal gland and in peripheral blood of C57BL/6, BALB/c and C57BL/6 x DBA2 (B6D2F1) mice, which were previously deemed to lack any melatonin secretion [33, 34]. However, melatonin secretion was weak, and its circadian pattern was shifted or bimodal, as compared to the prominent melatonin secretory patterns in C3H and CBA mice [33, 34]. Hence, our study could not robustly assess any effect of endogenous melatonin secretion on DEN carcinogenesis, or its impairment in clock-mutant mice. Nonetheless, the anticarcinogenic effects of melatonin against liver carcinogenesis remain an important issue. In rats, melatonin prevented chemically-induced hepatocarcinogenesis by reversing the oxidant-antioxidant imbalance during DEN exposure [35, 36], while in vitro exogenous melatonin induced apoptosis in cholangiocarcinoma cell lines [37]. Interestingly, a *Cry1/Cry2* double deficiency has been shown to both decrease mean level and suppress the circadian melatonin secretory pattern in C3H mice [38]. Accordingly, we would expect DEN-induced liver carcinogenesis to be reduced

in melatonin-proficient C3H mice, while being doubly enhanced both by circadian clock disruption and melatonin suppression in cryptochromes deficient C3H mice.

Decreased mRNA or protein expressions of *Cry1* and/or *Cry2*, were reported for human HCC, as well as for human cancers of the breast, endometrium, ovary, prostate, colon, pancreas, head-and-neck, brain, skin and blood cells [39-42]. Yet, the relevance of *Cry1/Cry2* deficiency for carcinogenesis was debated. A prior report showed that *Cry1/Cry2* deficiency promoted γ-radiation-induced lymphomas in mice [19]. This could result from increased cell cycling, since *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblasts were shown to proliferate faster than WT fibroblasts [43]. However, both of these latter findings [19, 43] contrasted with another report where *Cry1/Cry2* deficiency had no effect on γ-radiation-induced carcinogenesis and mortality in mice [44]. This latter finding was also in line with the lack of any up-regulation of the proto-oncogene *c-Myc* in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mouse liver and fibroblasts following radiation exposure, as well as the lack of modifications of cell cycle checkpoints, DNA repair, or apoptosis in *Cry* deficient fibroblasts [44]. Moreover, the combination of *Cry1/Cry2* deficiency with *p53* deletion protected mice from the early onset of γ-radiation-induced cancers and increased their median lifespan [45, 46].

However, our study unambiguously demonstrated a dramatic and unexpected CCA effect of DEN in mice with a *Cry1/Cry2* double deficiency. The magnitude of such effect was apparently unrelated to that of DEN toxicity. Thus, body weight loss, a marker of systemic toxicity, was least in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, a finding consistent with increased tolerability of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice to the alkylating drug cyclophosphamide [47]. In contrast, *Per2* mutant mice exposed to DEN displayed larger body weight loss as compared to their control [25]. Taken together, the results support CYP-mediated bioactivation of both DEN and cyclophosphamide was decreased in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ and enhanced in *Per2*$^{m/m}$ mice. Thus, *Cry1* and *Cry2* genes would be primarily involved in the promotion and progression stages, while having reduced

impact on initiation stage. This conclusion is also supported by the fact that *Cry1/Cry2* deficient mice displayed lower liver regeneration 72 hours after partial hepatectomy, associated with a high level of WEE1, which blocks G2-M transition [48].

DEN-induced liver carcinogenesis involves initial triggering of chronic inflammation [49, 50]. More specifically, CCA risk factors involve chronic biliary inflammation. According to several reports, pro-inflammatory cytokine IL-6 was up-regulated in cultured CCA cells, resulting in the activation of the JAK-STAT and NF-kB signalling pathways [51, 52]. IL-6 further increased *Mcl-1* through STAT-3 activation, and prevented apoptosis in CCA cells [51, 52]. In addition, IL-6 upregulation in CCA dysregulated the cell division cycle through the activation of P38 MAPK [53]. Indeed, several studies showed an important role of NF-kB activation in cholangiocarcinogenesis [54, 55]. The silencing of *Cry1* and *Cry2* enhanced lipopolysaccharide-induced inflammatory response through increased NF-κB and IL-6 activation in mouse hypothalamus, bone marrow macrophages, and fibroblasts [29, 56]. It has been shown that CRY1 protein bind to adenylyl cyclase and induces cAMP downregulation. In *Cry1*$^{-/-}$*Cry2*$^{-/-}$ cells, the inhibition of adenylyl-cyclase, leads to cAMP upregulation, PKA activation, P65 (a subunit of NF-kB complex) phosphorylation, and activation of NF-kB target genes such as IL-6 [56]. Thus, the increased biliary carcinogenesis found in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice exposed could result from an enhanced susceptibility to pre-existing and/or DEN-induced chronic biliary inflammation.

In contrast to the finding showing that *Per2* mutant mice are more susceptible to develop HCC [25], *Cry1*$^{-/-}$*Cry2*$^{-/-}$ deficient mice were more sensitive to DEN-induced CCA. WT mice subjected to left median bile duct ligation after 2 weeks of DEN exposure, then exposed to DEN, showed an increased susceptibility to develop CCA [4]. We suggest that the *Cry1/Cry2* double deficiency acts, like bile duct ligation, through accumulation of toxic bile acids in the

liver during the promotion stage, and thus would shift the cancer susceptibility of mice from HCC to CCA.

Although toxic bile acid accumulation increased liver susceptibility to develop HCC in animals, bile duct ligation-mediated toxic bile acid accumulation has also been shown to enhance CCA development [57, 58]. Bile acid accumulation locally altered proliferation and apoptosis (and even more so during cholestasis), which further predisposed to cholangiocarcinogenesis [59, 60]. Indeed, mice with bile duct ligation exposed to DEN displayed clearly accelerated CCA rather than HCC development [4]. Thus CCA occurred in 2.5 times as many mice with bile duct ligation as compared to those without such procedure (50% vs 20%), while the rate of HCC remained similar (20 %) in both groups [4]. Importantly, the CCA to HCC ratio was also largely positive and even greater in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ *vs* WT mice in our study, suggesting additional metabolic effects of the cryptochromes double deficiency. Interestingly the *Per1*$^{-/-}$*Per2*$^{-/-}$ mice, whose circadian phenotype closely resembles that of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animals, displayed elevated serum bile acid levels, while the circadian expression of key bile acid synthesis and transport genes (including Cyp7A1 and NTCP) was lost [61]. In aggregate, these reports, and our findings jointly support cholestasis and toxic bile acid accumulation to result from *Cry1/Cry2* double deficiency, and to shift DEN liver carcinogenesis from HCC toward CCA formation.

The current study has unambiguously validated that circadian clock disruption through *Cry1/Cry2* double deficiency more than doubled the incidence of diethylnitrosamine-induced liver cancers, with adequate statistical power. Eight times more cholangiocarcinomas were found in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ as compared to WT mice, while nearly 10% HCC occurred in both genotypes. We inferred that such major shift from HCC to CCA in the DEN liver

carcinogenesis model resulted from accumulated bile acids and cholestasis in the *Cry* double deficient mice.

References

Uncategorized References

1. Mancuso A. Management of hepatocellular carcinoma: Enlightening the gray zones. *World J Hepatol* 2013;**5**: 302-10.
2. Zabron A, Edwards RJ, Khan SA. The challenge of cholangiocarcinoma: dissecting the molecular mechanisms of an insidious cancer. *Dis Mod Mech* 2013;**6**: 281-92.
3. Lee JS, Grisham JW, Thorgeirsson SS. Comparative functional genomics for identifying models of human cancer. *Carcinogenesis* 2005;**26**: 1013-20.
4. Yang H, Li TW, Peng J, Tang X, Ko KS, Xia M, Aller MA. A mouse model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. *Gastroenterology* 2011;**141**: 378-88, 88 e1-4.
5. De Minicis S, Kisseleva T, Francis H, Baroni GS, Benedetti A, Brenner D, Alvaro D, Alpini G, Marzioni M. Liver carcinogenesis: rodent models of hepatocarcinoma and cholangiocarcinoma. *Dig Dis Liver* 2013;**45**: 450-9.
6. Vollmers C, Gill S, DiTacchio L, Pulivarthy SR, Le HD, Panda S. Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proc Natl Acad Sci U S A* 2009;**106**: 21453-8.
7. Schmutz I, Albrecht U, Ripperger JA. The role of clock genes and rhythmicity in the liver. *Mol Cell Endocrinol* 2012;**349**: 38-44.
8. Eckel-Mahan KL, Patel VR, Mohney RP, Vignola KS, Baldi P, Sassone-Corsi P. Coordination of the transcriptome and metabolome by the circadian clock. *Proc Natl Acad Sci U S A* 2012;**109**: 5541-6.
9. Fustin JM, Doi M, Yamada H, Komatsu R, Shimba S, Okamura H. Rhythmic nucleotide synthesis in the liver: temporal segregation of metabolites. *Cell Rep* 2012;**1**: 341-9.
10. Kornmann B, Schaad O, Reinke H, Saini C, Schibler U. Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* 2007;**72**: 319-30.
11. Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci* 2012;**35**: 445-62.
12. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. *Trends Cell Biol* 2013. 2014 Feb;24(2):90-9. doi: 10.1016/j.tcb.2013.07.002
13. Straif K, Baan R, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, Altieri A, Benbrahim-Tallaa L, Cogliano V. Carcinogenicity of shift-work, painting, and fire-fighting. *Lancet Oncol* 2007;**8**: 1065-6.
14. Wang F, Yeung KL, Chan WC, Kwok CC, Leung SL, Wu C, Chan EY, Yu IT, Yang XR, Tse LA. A meta-analysis on dose-response relationship between night shift work and the risk of breast cancer. *Ann Oncol* 2013;**24**: 2724-32.
15. Filipski E, King VM, Li X, Granda TG, Mormont MC, Liu X, Claustrat B, Hastings MH, Levi F. Host circadian clock as a control point in tumor progression. *J Natl Cancer Inst* 2002;**94**: 690-7.

16. Filipski E, Innominato PF, Wu M, Li XM, Iacobelli S, Xian LJ, Levi F. Effects of light and food schedules on liver and tumor molecular clocks in mice. *J Natl Cancer Inst* 2005;**97**: 507-17.

17. Li XM, Delaunay F, Dulong S, Claustrat B, Zampera S, Fujii Y, Teboul M, Beau J, Levi F. Cancer inhibition through circadian reprogramming of tumor transcriptome with meal timing. *Cancer Res* 2010;**70**: 3351-60.

18. Fu L, Pelicano H, Liu J, Huang P, Lee C. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* 2002;**111**: 41-50.

19. Lee S, Donehower LA, Herron AJ, Moore DD, Fu L. Disrupting circadian homeostasis of sympathetic signaling promotes tumor development in mice. *PLoS One* 2010;**5**: e10995.

20. Van Dycke KC, Rodenburg W, van Oostrom CT, van Kerkhof LW, Pennings JL, Roenneberg T, van Steeg H, van der Horst GT. Chronically alternating light cycles increase breast cancer risk in mice. *Curr Biol* 2015;**25**: 1932-7.

21. Innominato PF, Giacchetti S, Bjarnason GA, Focan C, Garufi C, Coudert B, Iacobelli S, Tampellini M, Durando X, Mormont MC, Waterhouse J, Levi FA. Prediction of overall survival through circadian rest-activity monitoring during chemotherapy for metastatic colorectal cancer. *Int J Cancer* 2012;**131**: 2684-92.

22. Ortiz-Tudela E, Mteyrek A, Ballesta A, Innominato PF, Levi F. Cancer chronotherapeutics: experimental, theoretical, and clinical aspects. *Handb Exp Pharmacol* 2013: 261-88.

23. van den Heiligenberg S, Depres-Brummer P, Barbason H, Claustrat B, Reynes M, Levi F. The tumor promoting effect of constant light exposure on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Life Sci* 1999;**64**: 2523-34.

24. Filipski E, Subramanian P, Carriere J, Guettier C, Barbason H, Levi F. Circadian disruption accelerates liver carcinogenesis in mice. *Mutat Res* 2009;**680**: 95-105.

25. Mteyrek A, Filipski E, Guettier C, Okyar A, Levi F. Clock gene Per2 as a controller of liver carcinogenesis. *Oncotarget* 2016;**7**: 85832-47.

26. Williams GM, Iatropoulos MJ, Wang CX, Ali N, Rivenson A, Peterson LA, Schulz C, Gebhardt R. Diethylnitrosamine exposure-responses for DNA damage, centrilobular cytotoxicity, cell proliferation and carcinogenesis in rat liver exhibit some non-linearities. *Carcinogenesis* 1996;**17**: 2253-8.

27. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005;**5**: 749-59.

28. van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 1999;**398**: 627-30.

29. Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW, Downes M, Evans RM. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature* 2011;**480**: 552-6.

30. Okamura H, Miyake S, Sumi Y, Yamaguchi S, Yasui A, Muijtjens M, Hoeijmakers JH, van der Horst GT. Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. *Science* 1999;**286**: 2531-4.

31. Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, et al. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* 2003;**278**: 41519-27.

32. Kettner NM, Voicu H, Finegold MJ, Coarfa C, Sreekumar A, Putluri N, Katchy CA, Lee C, Moore DD, Fu L. Circadian homeostasis of liver metabolism suppresses hepatocarcinogenesis. *Cancer Cell* 2016;**30**: 909-24.

33. Vivien-Roels B, Malan A, Rettori MC, Delagrange P, Jeanniot JP, Pevet P. Daily variations in pineal melatonin concentrations in inbred and outbred mice. *J Biol Rhythms* 1998;**13**: 403-9.

34. Li XM, Liu XH, Filipski E, Metzger G, Delagrange P, Jeanniot JP, Levi F. Relationship of atypical melatonin rhythm with two circadian clock outputs in B6D2F(1) mice. *Am J Physiol Regul Integr Comp Physiol* 2000;**278**: R924-30.

35. Dakshayani KB, Subramanian P, Manivasagam T, Essa MM, Manoharan S. Melatonin modulates the oxidant-antioxidant imbalance during N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *J Pharm Pharm Sci* 2005;**8**: 316-21.

36. Subramanian P, Mirunalini S, Dakshayani KB, Pandi-Perumal SR, Trakht I, Cardinali DP. Prevention by melatonin of hepatocarcinogenesis in rats injected with N-nitrosodiethylamine. *J Pineal Res* 2007;**43**: 305-12.

37. Laothong U, Hiraku Y, Oikawa S, Intuyod K, Murata M, Pinlaor S. Melatonin induces apoptosis in cholangiocarcinoma cell lines by activating the reactive oxygen species-mediated mitochondrial pathway. *Oncol Rep* 2015;**33**: 1443-9.

38. Yamanaka Y, Suzuki Y, Todo T, Honma K, Honma S. Loss of circadian rhythm and light-induced suppression of pineal melatonin levels in Cry1 and Cry2 double-deficient mice. *Genes Cells* 2010;**15**: 1063-71.

39. Mazzoccoli G, Piepoli A, Carella M, Panza A, Pazienza V, Benegiamo G, Palumbo O, Ranieri E. Altered expression of the clock gene machinery in kidney cancer patients. *Biomed Pharmacother* 2012;**66**: 175-9.

40. Lengyel Z, Lovig C, Kommedal S, Keszthelyi R, Szekeres G, Battyani Z, Csernus V, Nagy AD. Altered expression patterns of clock gene mRNAs and clock proteins in human skin tumors. *Tumour Biol* 2013;**34**: 811-9.

41. Mazzoccoli G, Colangelo T, Panza A, Rubino R, De Cata A, Tiberio C, Valvano MR, Pazienza V, Merla G, Augello B, Trombetta D, Storlazzi CT, et al. Deregulated expression of cryptochrome genes in human colorectal cancer. *Mol Cancer* 2016;**15**: 6.

42. Fu L, Kettner NM. The circadian clock in cancer development and therapy. *Prog Mol Biol Transl Sci* 2013;**119**: 221-82.

43. Destici E, Oklejewicz M, Saito S, van der Horst GT. Mammalian cryptochromes impinge on cell cycle progression in a circadian clock-independent manner. *Cell Cycle* 2011;**10**: 3788-97.

44. Gauger MA, Sancar A. Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res* 2005;**65**: 6828-34.

45. Ozturk N, Lee JH, Gaddameedhi S, Sancar A. Loss of cryptochrome reduces cancer risk in p53 mutant mice. *Proc Natl Acad Sci U S A* 2009;**106**: 2841-6.

46. Lee JH, Sancar A. Circadian clock disruption improves the efficacy of chemotherapy through p73-mediated apoptosis. *Proc Natl Acad Sci U S A* 2011;**108**: 10668-72.

47. Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch MP. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proc Natl Acad Sci U S A* 2005;**102**: 3407-12.

48. Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H. Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 2003;**302**: 255-9.

49. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005;**121**: 977-90.

50. Sun B, Karin M. Inflammation and liver tumorigenesis. *Front Med* 2013;**7**: 242-54.

51. Kobayashi S, Werneburg NW, Bronk SF, Kaufmann SH, Gores GJ. Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells. *Gastroenterology* 2005;**128**: 2054-65.

52. Isomoto H, Mott JL, Kobayashi S, Werneburg NW, Bronk SF, Haan S, Gores GJ. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. *Gastroenterology* 2007;**132**: 384-96.

53. Tadlock L, Patel T. Involvement of p38 mitogen-activated protein kinase signaling in transformed growth of a cholangiocarcinoma cell line. *Hepatology* 2001;**33**: 43-51.

54. Zhou Q, Gong Y, Huang F, Lin Q, Zeng B, Li Z, Chen R. Expression levels and significance of nuclear factor-kappaB and epidermal growth factor receptor in hepatolithiasis associated with intrahepatic cholangiocarcinoma. *Dig Surg* 2013;**30**: 309-16.

55. Dai J, Wang H, Dong Y, Zhang Y, Wang J. Bile acids affect the growth of human cholangiocarcinoma via NF-kB pathway. *Cancer Invest* 2013;**31**: 111-20.

56. Narasimamurthy R, Hatori M, Nayak SK, Liu F, Panda S, Verma IM. Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. *Proc Natl Acad Sci U S A* 2012;**109**: 12662-7.

57. Zhang Y, Hong JY, Rockwell CE, Copple BL, Jaeschke H, Klaassen CD. Effect of bile duct ligation on bile acid composition in mouse serum and liver. *Liver Int* 2012;**32**: 58-69.

58. Lozano E, Sanchez-Vicente L, Monte MJ, Herraez E, Briz O, Banales JM, Marin JJ, Macias RI. Cocarcinogenic effects of intrahepatic bile acid accumulation in cholangiocarcinoma development. *Mol Cancer Res* 2014;**12**: 91-100.

59. Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology* 2002;**122**: 985-93.

60. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. *Gastroenterology* 2013;**145**: 1215-29.

61. Ma K, Xiao R, Tseng HT, Shan L, Fu L, Moore DD. Circadian dysregulation disrupts bile acid homeostasis. *PLoS One* 2009;**4**: e6843.

Figure legends

Fig. 1. Systemic toxicity of DEN in WT and *Cry1$^{-/-}$Cry2$^{-/-}$* mice during the initiation, promotion, and progression carcinogenesis stages. (A) Relative body weight changes (mean ± SEM) in WT and *Cry1$^{-/-}$Cry2$^{-/-}$* mice throughout carcinogenesis stages. Weight loss was largest in WT mice (dark triangles) as compared to *Cry1$^{-/-}$Cry2$^{-/-}$* animals (open triangles). The daily DEN doses (mg/kg) are shown in italics, for corresponding exposure spans. (B)

Survival rate in WT (dark triangles) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (open triangles) mice exposed to DEN at ZT11. (C) Histogram of ALT serum activity (mean ± SEM) after DEN exposure of WT (dark box) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (open box) mice. (D) Serum level of AST (mean ± SEM) in WT (dark box) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (open box) mice. (E) Serum concentrations of ALP in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice on day 211. Mean (± SEM) ALT, AST and ALP serum levels in untreated control mice are represented by gray horizontal bars. * $p < 0.05$; ** $p < 0.01$

Fig. 2. Rest-activity and body temperature patterns and circadian parameters in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice throughout liver carcinogenesis. (A) Examples of rest-activity and body temperature patterns in a WT mouse (left) and in a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse (right) before DEN exposure. (B) Circadian acrophase changes for rest-activity (left panel) and body temperature (right panel) in WT (dark triangles) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (open triangles) animals before and during initiation (weeks 1-3), promotion (weeks 4-10), and progression (weeks 11-30) carcinogenesis stages. (C) Corresponding histograms of 24-h mean amplitudes with 95% C.L. for rest-activity (left panel) and body temperature (right panel) in WT (dark boxes) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (open boxes) mice before and throughout liver carcinogenesis stages. ** $p< 0.01$; *** $p\leq 0.001$.

Fig. 3. Examples of circadian activity and temperature rhythms records over 130 days in mice according to *Cry1*/*Cry2* double deficiency and DEN exposure. Representative double plot of 24-h rhythms in intraperitoneal temperature (A) and rest-activity (B) in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. The vertical black lines indicate exposure to DEN or vehicle (for 68 days).

Fig. 4. Representative histological slides of DEN-induced liver alterations in WT and *Cry1*/ *Cry2* double knock-out mice on the completing of the study (Day 211).
Typical macroscopic view of a deeply reorganized liver with macroscopic nodules (circled in white) arising in a WT mouse (**A**) and a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animal (**B**). Microscopic view (x10) of

untreated WT (**C**) or *Cry1*$^{-/-}$*Cry2*$^{-/-}$ control mice (**D**). Microscopic view (x10) of primary lesions (arrows) in WT (**E**) or *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (**F**). Detailed microscopic view (x20) of cholangiocarcinoma (**G**) and hepatocarcinoma **(H)**.

Fig. 5. DEN-induced liver cancer according to *Cry1/Cry2* double deficiency. (A) Number of tumor nodules per mouse liver in WT (black box) and in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (open box). (B) Average number of hepatocarcinomas (open box) and cholangiocarcinomas (grey box) in WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. ** $p < 0.01$; ***$p < 0.001$.