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# Circadian dysfunction induces NAFLD-related human liver cancer in a mouse model

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## Abstract

**Background & Aims:** Chronic circadian dysfunction increases the risk of non-alcoholic fatty liver disease (NAFLD)-related hepatocellular carcinoma (HCC), but the underlying mechanisms and direct relevance to human HCC have not been established. In this study, we aimed to determine whether chronic circadian dysregulation can drive NAFLD-related carcinogenesis from human hepatocytes and human HCC progression.

**Methods:** Chronic jet lag of mice with humanized livers induces spontaneous NAFLD-related HCCs from human hepatocytes. The clinical relevance of this model was analysed by biomarker,

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Conceptualization: LF, DDM; Methodology: LF, DDM, KDB, BBC, DLT, FL, JP, NMO; Investigation: JP, LF, BBC. FL, LY, XQ, AMM; Data analysis: LF, JP, NMO, LY, DLT, DDM, Bioinformatics: NMO, SG, CC, Pathology, AMM, JP, DLT; Writing-Original Draft: LF, DDM, NMO, JP, DLT, FL, KDB; Revision: LF, NMO, DDM, KDB; Supervision: LF; Funding Acquisition: LF, DDM, KDB.

Supplementary data

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Conflict of interest

The authors declare no conflicts of interest.

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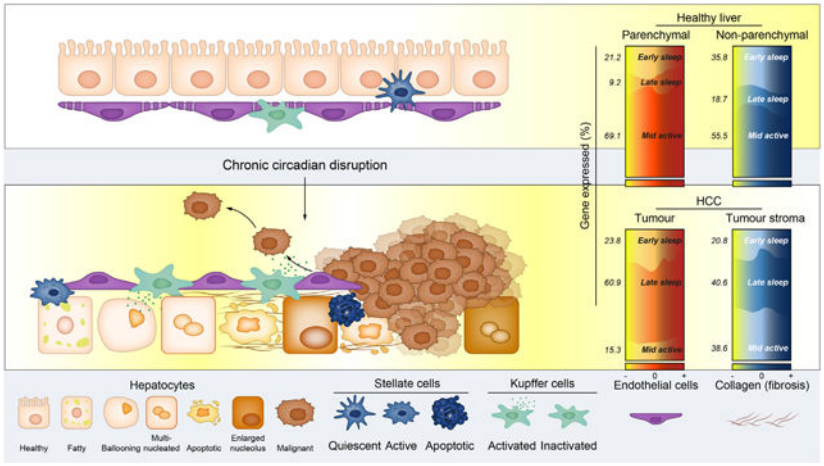
pathological/histological, genetic, RNA sequencing, metabolomic, and integrated bioinformatic analyses.

**Results:** Circadian dysfunction induces glucose intolerance, NAFLD-associated human HCCs, and human HCC metastasis independent of diet in a humanized mouse model. The deregulated transcriptomes in necrotic-inflammatory humanized livers and HCCs bear a striking resemblance to those of human non-alcoholic steatohepatitis (NASH), cirrhosis, and HCC. Stable circadian entrainment of hosts rhythmically paces NASH and HCC transcriptomes to decrease HCC incidence and prevent HCC metastasis. Circadian disruption directly reprogrammes NASH and HCC transcriptomes to drive a rapid progression from hepatocarcinogenesis to HCC metastasis. Human hepatocyte and tumour transcripts are clearly distinguishable from mouse transcripts in non-parenchymal cells and tumour stroma, and display dynamic changes in metabolism, inflammation, angiogenesis, and oncogenic signalling in NASH, progressing to hepatocyte malignant transformation and immunosuppressive tumour stroma in HCCs. Metabolomic analysis defines specific bile acids as prognostic biomarkers that change dynamically during hepatocarcinogenesis and in response to circadian disruption at all disease stages.

**Conclusion:** Chronic circadian dysfunction is independently carcinogenic to human hepatocytes. Mice with humanized livers provide a powerful preclinical model for studying the impact of the necrotic-inflammatory liver environment and neuroendocrine circadian dysfunction on hepatocarcinogenesis and anti-HCC therapy.

## Graphical abstract




### Keywords

hepatocellular carcinoma; circadian disruption; non-alcoholic steatohepatitis (NASH); cirrhosis; circadian transcriptomes

## Introduction

The incidence of hepatocellular carcinoma (HCC) has more than tripled in the US since the 1980s, with HCV infection as the primary driver.[1] The implementation of direct anti-HCV agents has decreased virus-induced HCC in recent years. However, this decline is

counterbalanced by the increasing impact of obesity-related NAFLD. The escalating global obesity pandemic means that NAFLD will likely be the leading cause of HCC by 2030.[2]

NAFLD progresses step-wise to NASH, cirrhosis, a strong HCC risk factor associated with all HCC aetiologies, and then HCC. However, about 20–30% of NAFLD-related HCC occurs without cirrhosis, suggesting a unique molecular pathogenesis for NAFLD-induced HCC.[1] No effective clinical interventions are currently available to address NAFLD-induced hepatocarcinogenesis. [3] The lack of pre-clinical animal models spontaneously developing NAFLD-related HCC as observed in obese humans significantly impairs our understanding of this disease.[4]

Evolutionary adaptation to environmental light/dark changes dictates that most physiological processes in mammals follow a circadian rhythm generated by an endogenous clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus.[5] The SCN clock rhythmically couples behavioural rhythms with endogenous neuroendocrine outputs, which together control peripheral tissues via cell signalling. At the molecular level, the clock is operated by feedback loops of circadian genes capable of generating circadian rhythm of gene expression via a cell-autonomous mechanism.[5] Circadian gene deficiencies increase whole body or tissue-specific adiposity, metabolic syndrome, NAFLD, and hepatocarcinogenesis in rodents.[6–8] However, the persistent free-running of the endogenous clock in rodents is associated with increased oxidative stress, metabolic disruption, neurodegeneration, immune suppression, and clock gene deregulation in peripheral organs, with the adverse effects more evident in diurnal than in nocturnal animals.[9–11] Thus, the stable coupling of the endogenous rhythm to environmental light cues is required for optimal health in mammals.

Circadian entrainment in mammals is controlled by complex cell signalling networks mediating the response of the SCN to external cues and the crosstalk of the SCN with other brain centres. These couple behavioural, body temperature, and feeding rhythms with neuroendocrine circadian outputs, which together entrain peripheral oscillators and peripheral cues to establish adaptive synchrony in internal physiology.[12] Irregular light exposure phase-shifts the central clock to temporarily uncouple behavioural and feeding rhythms from neuroendocrine signalling. Chronic phase-shifts of the SCN induce neuroendocrine dysfunction which disrupts the peripheral clocks independent of circadian gene deficiencies.[13–15] Thus, circadian homeostasis in free-living humans is a physiological function not regulated at a cell-autonomous level.

The increased risk of NAFLD-related HCC in humans is coupled with epidemics of both obesity and chronic circadian disruption.[16–18] We reported previously that chronic jet lag following a human 8-hour shiftwork schedule induces obesity, neuroendocrine dysfunction, hyperglycaemia, hyperinsulinemia, dyslipidaemia, intrahepatic cholestasis, NAFLD, and NAFLD-related HCC in wild-type (WT) mice.[7,8,19] Here we show that chronic jet lag also induces NAFLD-related HCC from human hepatocytes.

## Materials and methods

### Animal studies

Animal experiments followed the ARRIVE guideline for research on animals and are approved by the Institutional Animal Care and Use Committee.

### Humanized mice

The generation of humanized transgene-free *Il2rg*$^{-/-}$;*Rag2*$^{-/-}$;*Fah*$^{-/-}$ (TIRF) mice is described in the supplementary materials and methods.[20]

### Chronic jet lag

Automatically advancing (Mondays to Wednesdays) and delaying (Thursdays to Sundays) the time of ceiling light-off for 8-hours in the same animal room drives chronic 8-hour rotating phase-shifts in the SCN of mice, mimicking an 8-hour shiftwork schedule of security staff in our institute (supplementary materials and methods).[7,8,19]

### Mouse survival studies

Animal survival studies follow the Morton and Griffiths guide from 8–60 weeks of age as described in the supplementary materials and methods.[7,8,19]

### Mouse serum, tissue, and tumour isolation

Mouse serum, livers, and tumours were collected at zeitgeber time (ZT) 2, 10, and 18 as described in the supplementary materials and methods.[7,8,19]

### ELISA

ELISA analysis of serum biomarkers is described in the supplementary materials and methods.

### Liver and tumour genotyping

Humanized livers and HCCs were genotyped as described in the supplementary materials and methods.

### Histology and pathological diagnosis

The histological and immunohistochemistry (IHC) analyses of humanized livers and tumours were performed as described in the supplementary materials and methods.[7,19]

### Transcriptome analysis

RNA isolation, reverse-transcription PCR, and RNA sequencing are described in the supplementary materials and methods.[19]

### Metabolomic analysis

Metabolomic analysis of free fatty acids (FFAs) and bile acids (BAs) is described in the supplementary materials and methods.[19]

### Bioinformatic and statistical analyses

Bioinformatic and statistical analyses were performed as described in the supplementary materials and methods.[19] $p$ values <0.05 were considered statistically significant in all analyses.

## Results

### Circadian dysfunction induces NAFLD-related human HCCs

Mice with humanized livers were generated by transplanting normal human hepatocytes into immunodeficient TIRF mice (Fig. 1A–B). The lack of *Fah* induces toxic fumarylacetoacetate accumulation in mouse hepatocytes, leading to liver failure and death in TIRF mice. Thus, these mice require 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) in drinking water to block fumarylacetoacetate accumulation. Periodic NTBC withdrawal after human hepatocyte transplantation induces the death of $Fah^{-/-}$ mouse hepatocytes and liver regeneration from $Fah^{+/+}$ human hepatocytes. This establishes humanized livers in which human hepatocytes are supported by mouse vascular and neuroendocrine systems and non-parenchymal cells in a natural liver microenvironment (Fig. 1C).[20]

Male humanized TIRF mice were group-housed, fed chow and periodically treated by NTBC withdrawal. They were then assigned to control, chronically jet-lagged, and jet-lagged-and-cholic acid-fed groups and monitored until 60 weeks of age (Table S1). The cholic acid diet was included to study the role of exogeneous toxic BAs in jet lag-induced hepatocarcinogenesis. Chronic jet lag followed a weekly human 8-hour shiftwork schedule (Fig. S1).[7,8,19] Compared to control mice kept in steady 24-hour light/dark (LD) cycles, mice in the two jet-lagged groups showed significantly reduced lifespan and increased cirrhosis, jaundice, kidney failure, and hepatocarcinogenesis. HCC was the only cancer identified since the severe hepatic necrotic-inflammatory microenvironment in TIRF mice induced rapid hepatocarcinogenesis prior to tumour detection in other tissues (Fig. 1D,E; Table 1).

Serum and livers from healthy control mice and HCCs and necrotic-inflammatory livers from terminally ill mice in control and jet-lagged groups were isolated at ZT 2 (early sleep phase), 10 (late sleep phase), and 18 (mid of active phase), the three distinct physiological statuses most sensitive to neuroendocrine circadian disruptions over a 24-hour period.[7,8,19] Small liver fragments and HCCs were individually genotyped for human (*CTNNB1*, *FAH*, and Alu repeats) and mouse (*Nr1h4*) genes to identify humanized healthy and necrotic-inflammatory livers and HCCs. Liver fragments and HCCs with a ratio of human genes to mouse NR1H4 signal ≥1 were considered humanized livers and HCCs, comprising human hepatocytes and mouse non-parenchymal cells in livers and malignant human hepatocytes and mouse tumour stroma in HCCs, respectively (Fig. 2A and Fig. S2). Since fewer control mice developed humanized HCCs, four tumour-bearing jet-lagged mice were re-entrained for 3 weeks to stable 24-hour LD cycles for additional humanized HCC and necrotic-inflammatory liver isolation. Humanized livers and tumours isolated under the entrained

condition were termed control (Ctrl), while those isolated under the jet-lagged condition were termed chronically jet-lagged (CJ) in the following mechanistic studies.

About 41% of HCCs (126/307) and 77% of liver fragments (112/146) genotyped were found to be humanized. Jet lag decreased latency but increased incidence and growth rate of both mouse and humanized HCCs (Fig. 2B,D; Table 1). Strikingly, jet lag also induced metastasis of humanized HCCs (Fig. 2E,F). Humanized HCC incidence was independent of the rate of human hepatocyte repopulation and gender or age of human hepatocyte donors (Fig. 2G,H; Table 1 and Table S1). Cholic acid intake did not significantly affect survival or humanized HCC incidence, although it did increase tumour size and cirrhosis-related death (Figs 1D and 2B–D; Table 1). Overall, we conclude that chronic circadian dysfunction is an independent carcinogen for human hepatocytes.

Serum biomarker analyses revealed that humanized TIRF mice developed hyperglycaemia and hyperinsulinemia prior to human HCC detection. They also displayed dramatically elevated serum biomarkers of hepatic damage, inflammation, and fibrosis, including alanine aminotransferase, TNFα, and IL-6, which are frequently observed in human patients with NASH and/or cirrhosis (Fig. 2I).[21,22]

Analyses of Oil Red O (ORO)- and FAH-stained liver sections revealed that jet-lagged TIRF mice developed NAFLD in $Fah^{+/+}$ humanized but not $Fah^{-/-}$ mouse liver sections (Fig. 3A and Fig. S3A). NAFLD development was coupled with decreased FAH expression in human hepatocytes, dramatically increased hematopoietic cell and macrophage infiltration, and the appearance of severe fibrosis in humanized livers prior to HCC detection (Fig. 3B–D and Fig. S3B–3C). IHC also detected highly heterogeneous expression of human GPC-3, CTNNB1, and c-MYC in humanized NASH livers and HCCs as observed in human NASH and HCCs, while FAH expression was undetectable in most humanized HCCs (Fig. 3E and Fig. S4). Together, the serum biomarker and pathological studies demonstrated that the pathogenesis of humanized HCCs in jet-lagged TIRF mice shares multiple commonalities with NAFLD-induced HCCs in humans, including glucose intolerance, abnormal hepatic fat accumulation, inflammation and fibrosis, and histologic features of NASH before HCC initiation.[1]

## Circadian disruption reprogrammes parenchymal and non-parenchymal transcriptomes

We examined molecular mechanisms driving circadian dysfunction-induced carcinogenesis in human hepatocytes by RNA sequencing using mRNAs prepared from humanized livers and HCCs under both entrained and jet-lagged conditions (Fig. S5A,B).

Bioinformatic analyses identified large numbers of differentially expressed genes (DEGs) in human (hepatocytes) and mouse (non-parenchymal cells/HCC stroma) transcriptomes. In stable 24-hour LD cycles, about 9% of protein-coding genes in human hepatocytes and 15% of those in mouse non-parenchymal cells displayed rhythmic expression in healthy humanized livers (Fig. 4A and Fig. S5C; Table S2).

About 22% and 10% of human protein coding genes and 28% and 26% of mouse protein coding genes were deregulated in humanized NASH and HCCs under the entrained

condition, respectively (Fig. 4B and Fig. S5D). Up to 73% of human (3,432/4,678) and mouse (4,197/5,491) DEGs in NASH but only 22% of human (460/2,102) and 18% of mouse (980/5,424) DEGs in HCCs were rhythmically expressed (Fig. 4A,B and Fig. S5C,D). The rhythmic expression of human DEGs in control NASH and HCCs followed a profile similar to that in healthy humanized livers, while the expression profile of mouse DEGs in HCC stroma was dramatically different from that in normal and NASH liver stroma, indicating a significant difference in cell components in the liver and HCC stroma (Fig. 4A,B and Fig. S5C,D).

Chronic jet lag strongly and differentially shifted human transcriptomes in NASH and HCCs leading to >1.5-fold increase in total DEGs and >4-fold increase in time-dependent DEGs in tumours, with changes in the numbers and redistribution of human DEGs most evident in both early and late sleep phases in NASH and in the late sleep phase in HCCs (Fig. 4A,B). Jet lag also significantly increased time-dependent DEGs in the sleep phase in NASH and HCC stroma transcriptomes (Fig. S5C,D; Table S2). Overall, these findings suggest that hepatocarcinogenesis is associated with progressive dedifferentiation of hepatocytes and dampening and uncoupling of parenchymal and non-parenchymal transcriptomes.

Over-representation analysis defined deregulated pathways driving hepatocarcinogenesis in both human and mouse transcriptomes (Fig. 4C–F and Fig S6–11; Table S3–S5). As expected, cholic acid intake did not significantly impact the HCC gene signature in tumours or tumour stroma (Fig. S6–7). Thus, chronic circadian dysfunction played an independent role in molecular carcinogenesis of human hepatocytes.

Compared to that in healthy humanized livers, the deregulated transcriptome in humanized NASH displayed strong and time-dependent activation of oxidative phosphorylation, deregulation of glucose, xenobiotic, BA, cholesterol, and fatty acid metabolism, as well as adipogenesis, hypoxia, and inflammatory responses, in hepatocytes. These effects were coupled by oncogenic activation and deregulation of the G2/M checkpoint and p53 pathway in both parenchymal and non-parenchymal cells (Fig. 4C and Fig. S8). Jet lag suppressed oxidative phosphorylation but promoted epithelial-mesenchymal transition (EMT) in hepatocytes in NASH, and metabolic reprogramming, apoptosis, hypoxia, inflammatory responses, and deregulation of checkpoint and oncogenic and p53 signalling in both hepatocytes and non-parenchymal cells (Fig. 4D and Fig. S9; Table S4–S5).

Under the entrained condition, most deregulated pathways in the human tumour transcriptome displayed robust circadian rhythms, especially those promoting metabolic reprogramming and hepatocyte malignant transformation, such as over-activation of hypoxia, apoptosis, angiogenesis, tumour-associated inflammation, oncogenic signalling, and EMT but suppression of DNA repair, damage responses, the G2/M checkpoint, and p53 signalling (Fig. 4E and Fig. S10). The mouse tumour stroma transcriptome displayed a hypoxic, angiogenic, and immune suppressive signature together with dysregulation of p53, damage responses, glycolysis, and apoptosis, and activation of stroma-related EMT which is associated with tumour progression and the resistance to immune therapy in humans (Fig. 4E and Fig. S10; Table S4–S5).[23]

Compared to the transcriptomes in healthy humanized livers, jet lag further shifted HCC transcriptomes to constitutively activate EMT, metabolic reprogramming, hypoxia, and inflammatory and oncogenic signalling, and further suppress checkpoint and DNA repair pathways. The mouse tumour stroma transcriptome showed a strong immune suppressive and hypoxic signature together with constitutive activation of stroma-related EMT (Fig. 4F and Fig. S11; Table S4–S5).

Surprisingly, oestrogen and androgen pathways displayed a robust circadian rhythm in parenchymal and non-parenchymal cells in healthy and NASH livers and were among the top deregulated pathways throughout NAFLD-induced hepatocarcinogenesis (Figs S6–11; Table S4–S5).

## Chronic jet lag disrupts the liver clock and serum and hepatic prognostic biomarkers

Reverse-transcription PCR revealed that the expression of human and mouse core circadian genes displayed a coupled circadian profile in healthy humanized livers under the entrained condition but was progressively deregulated and uncoupled in NASH and HCCs under both entrained and jet-lagged conditions (Fig.5A and Fig. S12). The dysregulation of clock genes in NASH and HCCs was associated with the over-expression of proto-oncogenes *c-Myc* and *c-Fos*, suppression of *Nr1h4* (encoding the nuclear BA receptor FXR) and *Fah*, and upregulation of the human proto-oncogene *FoxM1* in hepatocytes in NASH and mouse *FoxM1* in HCC stroma. *Nr1i3* (encoding nuclear xenobiotic receptor CAR) was upregulated in tumours but suppressed in tumour stroma, coupled with differential deregulation of cytochrome P450 genes for drug metabolism, such as suppression of *CYP2B6* but upregulation of *CYP3A4* in tumours and inhibition of both *Cyp2b10* and Cyp3a11 in tumour stroma (Fig. 5A and Fig. S12).

FFA and BA metabolic pathways were among the top deregulated gene pathways throughout NAFLD-induced hepatocarcinogenesis in human hepatocytes (Fig. 4C–F and Figs S6–S11). Thus, BAs and FFAs could be prognostic biomarkers for NAFLD-induced hepatocarcinogenesis. We studied serum and hepatic FFAs and BAs in TIRF mice with humanized NASH and HCCs by metabolomic profiling. All FFAs and BAs studied displayed distinct circadian oscillation patterns in serum and livers of healthy control humanized TIRF mice, which were deregulated in mice with humanized NASH and HCCs (Fig. 5B and Fig. S13). Overall, under the entrained condition, mice with NASH displayed decreased plasma but increased hepatic levels of most FFAs, especially in the early sleep and active phases, whereas BAs displayed time-dependent and BA-specific deregulated patterns in serum and NASH (Fig. S13). Mice with humanized HCCs showed increased plasma levels of most FFAs in the late sleep phase and most BAs in the active phase but dramatically decreased levels of most FFAs and BAs in tumours at all times studied (Fig. 5B and Fig. S13). Chronic jet lag significantly shifted the profiles of all FFAs and BAs in serum, NASH, and HCCs coupled with changes in FFA and BA pathways in NASH and HCC transcriptomes (Fig. 5B and Fig. S13). However, a panel of BAs were persistently elevated in serum, NASH, and HCCs, especially under jet-lagged conditions, throughout hepatocarcinogenesis, such as the toxic chenodeoxycholic, deoxycholic, and lithocholic acids, and glycochenodeoxycholic, glycodeoxycholic, and

taurochenodeoxycholic/taurodeoxycholic acids, which are known biomarkers of liver injury and acute failure (Fig. 5B).[24] Thus, these BAs are potential prognostic biomarkers of NAFLD-induced HCC.

### Clinical relevance of humanized NASH and HCCs

Comparative analyses of the humanized NASH transcriptome with those in human non-surgical NASH biopsies and cirrhosis revealed that the humanized NASH transcriptome significantly overlapped with both human NASH and cirrhosis transcriptomes (Fig. 6A,B; Table S6). However, it overlapped better with that of human NASH than with human cirrhosis under the entrained condition, but much more strongly with the human cirrhosis transcriptome under the jet-lagged condition (Fig. 6A,B). The analysis of 1,085 DEGs unique to the jet-lagged humanized NASH transcriptome defined a prognostic signature predicting increased NASH to cirrhosis progression, which is highly enriched in pathways promoting chronic inflammation, p53 response, fibrosis, EMT, and oncogenic, androgen, and oestrogen signalling, and suppressing G2/M checkpoint and DNA repair (Fig. 6C and Fig. S14; Table S7).

We found a strikingly high similarity between reported human HCC transcriptomes (tumour plus stroma) and our combined humanized HCC transcriptomes derived from both human and mouse DEGs. There was also a high similarity between the human HCC transcriptomes and those of the separated components of humanized HCCs, namely the human tumour and mouse tumour stroma transcriptomes derived from human and mouse DEGs in all humanized HCCs studied, respectively (Fig. 6D).

We then defined three human HCC transcriptome clusters based on patient survival (Fig. 6E), the 722 DEGs specific to human HCC gene Cluster 1 associated with the best survival, and the 1,178 DEGs unique to human HCC gene Cluster 3 associated with the worst survival (Fig. S15A). We also identified 1,203 and 1,860 DEGs specific to humanized HCCs under entrained or jet-lagged conditions, respectively (Fig. 6F and Table S7). Comparative bioinformatic analyses revealed that DEGs specific to humanized HCCs under the entrained condition overlapped significantly with DEGs unique to human HCC gene Cluster 1 but not those unique to human HCC gene Cluster 3. Whereas DEGs unique to jet lagged humanized HCCs only overlapped significantly with those specific to human HCC gene Cluster 3 but not those in human HCC gene Cluster 1 (Fig. 6G). Thus, over-representation analysis of the 1,203 DEGs unique to control humanized HCCs defined a prognostic signature predicting better survival (Fig. 6H and Fig. S15), while the 1,860 DEGs specific to jet-lagged humanized HCCs generated a prognostic signature predicting HCC progression and metastasis, which is highly enriched in oncogenic signalling, cancer metabolism, immune invasion, hypoxia, angiogenesis, oestrogen and androgen responses, and epithelium-stroma interactions (Fig. 6I and Fig. S15; Table S7).

## Discussion

Most animal models of NASH-related HCC, either induced by diet, gene ablation, or carcinogens, or the combinations of these manipulations, do not display the full metabolic and molecular features of human NASH or HCCs, although they may exhibit a histologic

appearance resembling those in human NASH and HCC.[4] Chronically jet-lagged TIRF mice spontaneously developed NAFLD-induced HCC in human hepatocytes, following the same aetiology and pathophysiological/molecular pathways as those in obese humans. This establishes the unique strength of this model for studying the mechanism of and therapeutic options for NAFLD-related HCC (Figs 1–6).

Humans need 8 days to re-establish circadian homeostasis after trans-meridian travels crossing eight time zones from west to east and 4 days to do so after crossing eight time zones from east to west.[25] The weekly 8-hour shiftwork schedule induces persistent circadian desynchronization like trans-meridian travels regardless of weekend schedules.[26] Circadian dysfunction abolishes neuroendocrine homeostasis to disrupt peripheral clocks independent of circadian gene deficiencies in humans and animals.[8,13,14,19,27–30] In WT mice, one cycle of back-and-forth 8-hour jet lag completely disrupts peripheral oscillators,[7] but the behavioural and feeding rhythms of these mice are still coupled to external light cues after 15 consecutive cycles of jet lag due to the light masking effects on the central clock.[8] Thus, behavioural rhythm is only coupled to endogenous neuroendocrine homeostasis under the entrained condition, and that neuroendocrine circadian dysfunction drives jet lag syndrome.[31]

In control healthy TIRF mice, the molecular clock in human hepatocytes is stably entrained to the host nocturnal time cues following the same pattern as observed in WT mouse livers under the entrained condition,[19] but was progressively disrupted and uncoupled from that in mouse non-parenchymal cells in NASH and HCCs, especially under jet-lagged condition (Fig. 5A). These findings agree with the notion that *in vivo*, the circadian homeostasis of neuroendocrine signaling plays a dominant role in maintaining circadian homeostasis in peripheral tissues.

Our transcriptome studies reveal that spontaneous hepatocarcinogenesis is driven by thousands of DEGs expressed in a cell type-, time-, and disease stage-specific manner in the liver. Stable entrainment of hosts can efficiently couple NASH and HCC transcriptomes to external circadian time cues, which significantly reduces the risk of NASH to cirrhosis progression and HCC initiation and metastasis. Chronic circadian disruption directly reprogrammes thousands of DEGs in NASH and HCC transcriptomes to drive a full-range activation of hallmarks of cancer, resulting in a rapid progression from NAFLD to hepatocarcinogenesis and then HCC metastasis (Fig. 1–6, Figs S5–11, and S14,15). Human HCCs are characterized by remarkable inter-patient, inter-tumoral, and intra-tumoral heterogeneities and the lack of oncogene loops for therapeutic targeting.[1] Our striking discoveries not only provide a molecular explanation for the powerful impact of circadian homeostasis in tumour suppression but will also have important therapeutic implications for the future prevention and treatment of HCC in humans.

The oncogenic impacts of circadian dysfunction in NASH include metabolic reprogramming, sustained cell proliferation and inflammation, evading growth suppressors, DNA surveillance, and programmed cell death, increased hepatocyte trans-differentiation, and dysregulation of extracellular matrix. Persistent circadian dysfunction promotes NASH to cirrhosis progression and stimulates formation of preneoplastic niches characterized by

tumour-promoting inflammation, accelerated angiogenesis, and activation of stroma-related EMT (Fig. 1–6, Figs S5–11, and S14,15). In addition to previously identified dysregulation of sympathetic, leptin, insulin, and bile acid signalling,[7,8,19] our transcriptome analysis revealed that jet lag also induces deregulation of oestrogen, androgen, WNT/β-catenin, TGFβ, PI3K-AKT-mTOR, interferon, hedgehog, IL6-JAK-STAT3, Notch, IL2-STAT5, and TNFα-NF-κB signalling in the liver to promote hepatocarcinogenesis (Figs S14,15; Table S2–5 and S7).

High-throughput transcriptome studies have generated various prognostic signatures of human NASH, cirrhosis, and HCC but none have achieved clinical implementation.[32] One of the biggest challenges in human HCC studies is that they are usually based on a snapshot of gene expression pattern at a fixed time under a special physiological condition. It is striking that only 30% of DEGs in human non-surgical and surgical NASH transcriptomes share similar expression patterns, and that surgical procedures dramatically change the expression of genes controlling tumour suppression, immune responses, cell signalling, metabolism, and proliferation (Fig. S16; Table S8). The powerful circadian impacts on both humanized NASH and HCC transcriptomes found in our studies strongly justify the importance of preclinically relevant animal models for studying both HCC biology and HCC-host interactions under dynamic physiological conditions.

Our studies identified a panel of BAs as potential prognostic biomarkers of NAFLD-related hepatocarcinogenesis and revealed that the circadian profiles of serum and hepatic biomarkers can be readily changed by circadian disruption at all disease stages studied (Figs 5B and Fig. S13). Thus, circadian dysfunction can substantially complicate attempts to define HCC prognostic markers and interfere with HCC diagnosis and treatment.

HCC is a prototypical inflammation-driven cancer, arising exclusively in the setting of chronic inflammation and exhibits strong resistanceto anticancer therapy.[1] Despite recent progress, anti-HCC therapy is challenged by the lack of efficient early diagnostic and prognostic biomarkers and combination treatment strategies.[33] In jet-lagged TIRF mice, the strikingly accelerated rate of spontaneous carcinogenesis from human hepatocytes does not appear related to age or gender of hepatocyte donors (Figs 1 and 2; Table S1), suggesting a key role of dysregulation of liver stroma in hepatocarcinogenesis. Thus, humanized TIRF mice also act as an excellent pre-clinical model, providing unique insights into the severity of the necrotic-inflammatory liver environment in HCC initiation, progression, and therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability statement

Data are available upon request from the corresponding author. GEO accession number: GSE205881.

## Abbreviations

| | |
|---|---|
| **BA** | bile acid |
| **DEG** | differentially expressed gene |
| **EMT** | epithelial-mesenchymal transition |
| **FFA** | free fatty acid |
| **HCC** | hepatocellular carcinoma |
| **IHC** | immunohistochemistry |
| **LD** | light/dark |
| **NAFLD** | non-alcoholic fatty liver disease |
| **NASH** | non-alcoholic steatohepatitis |
| **NTBC** | 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione |
| **ORO** | Oil Red O |
| **SCN** | suprachiasmatic nucleus |
| **TIRF mice** | transgene-free Il2rg$^{-/-}$;Rag2$^{-/-}$;Fah$^{-/-}$ mice |
| **WT** | wild-type |
| **ZT** | zeitgeber time |

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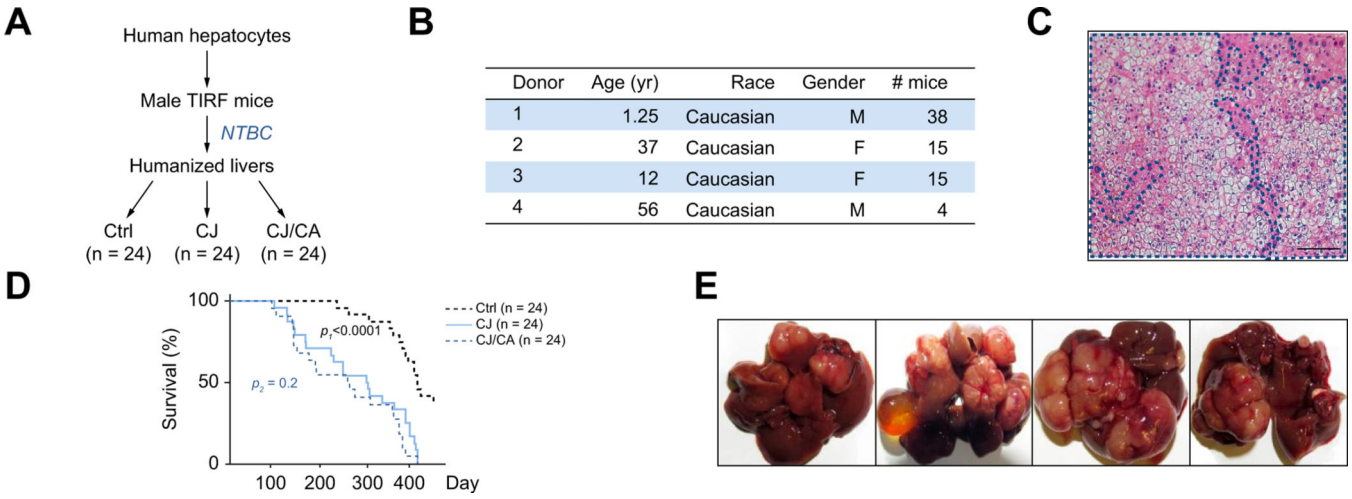
## Impact and implications

Human epidemiological studies have linked chronic circadian dysfunction to increased hepatocellular carcinoma (HCC) risk, but direct evidence that circadian dysfunction is a human carcinogen has not been established. Here we show that circadian dysfunction induces non-alcoholic steatohepatitis (NASH)-related carcinogenesis from human hepatocytes in a murine humanized liver model, following the same molecular and pathologic pathways observed in human patients. The gene expression signatures of humanized HCC transcriptomes from circadian-disrupted mice closely match those of human HCC with the poorest prognostic outcomes, while those from stably circadian entrained mice match those from human HCC with the best prognostic outcomes. Our studies establish a new model for defining the mechanism of NASH-related HCC and highlight the importance of circadian biology in HCC prevention and treatment.

## Highlights

- Circadian dysfunction promotes NAFLD-induced carcinogenesis in human hepatocytes.
- Chronic jet lag shifts NASH and HCC transcriptomes to activate hallmarks of cancer.
- Circadian dysfunction reprograms transcriptomes in HCC to stimulate tumour metastasis.
- Circadian disruption shifts prognostic biomarkers to interfere with HCC diagnosis.
- Circadian homeostasis is strongly relevant to future precision oncology.




| Donor | Age (yr) | Race | Gender | # mice |
|---|---|---|---|---|
| 1 | 1.25 | Caucasian | M | 38 |
| 2 | 37 | Caucasian | F | 15 |
| 3 | 12 | Caucasian | F | 15 |
| 4 | 56 | Caucasian | M | 4 |

**Fig. 1. Chronic jet lag increases HCC incidence in humanized TIFR mice.**

(A) A schematic representation of humanized TIRF mice. Control (Ctrl): maintained in steady 24-hour LD cycles. Chronically jet-lagged (CJ): chronically treated with a human 8-hour shiftwork schedule. CJ/CA: chronically jet-lagged and fed with a chow diet containing 2% cholic acid. (B) The summary of human hepatocyte donors. (C) A H&E-stained section of healthy h-liver (circled by dashed blue lines) shows less eosinophilic human hepatocytes readily distinguishable from more acidic mouse cells in a natural liver microenvironment (scalebar: 100 μm). (D) The survival of humanized TIRF mice in each group. $p1$: Ctrl *vs.* CJ; $p2$: CJ *vs.* CJ/CA, Kaplan-Meier Statistics, *$p<0.05$. (E) Representative images of HCCs found in TIRF mice. h-liver, humanized liver; HCC, hepatocellular carcinoma; TIRF mice, transgene-free *Il2rg*$^{-/-}$;*Rag2*$^{-/-}$;*Fah*$^{-/-}$ mice.

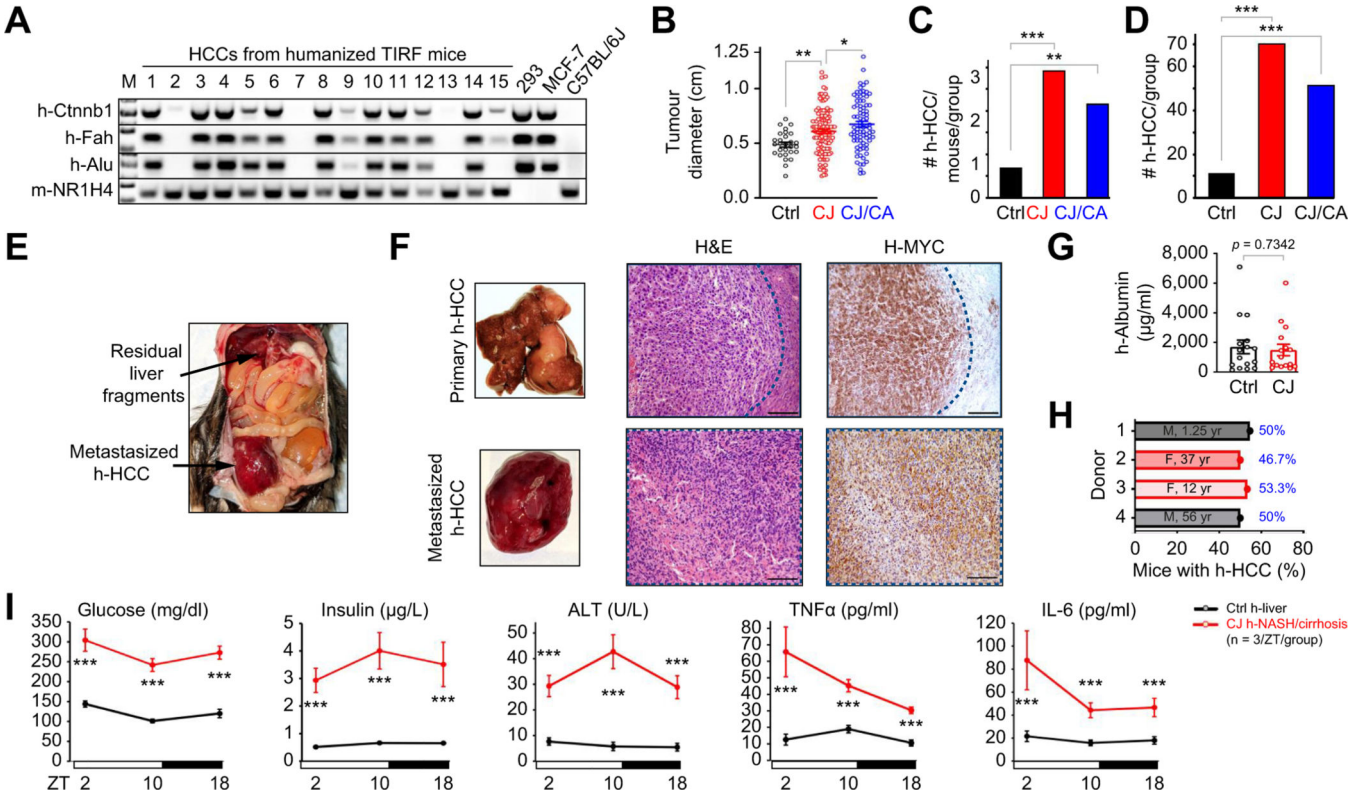



**Fig. 2. Circadian dysfunction induces humanized HCCs.**

(A) A representative image of PCR genotyping of HCCs (293: human embryonic kidney cells; MCF-7: human breast cancer cells; C57BL/6J: C57BL/6J mouse tails). (B) The summary of the sizes of HCCs in each group. (C-D) The incidence of h-HCC in each group. (E) A gross image shows a h-HCC metastasized from the liver to the lumbar vertebrae in a jet-lagged TIRF mouse. (F) Gross images (left panel) of a primary h-HCC (top) metastasized to the lumbar bone (bottom) in a jet-lagged TIRF mouse. H&E-stained sections (central panel) show a primary (top) and a metastasized (bottom) h-HCC. IHC sections (right panel) show strong human MYC expression in both primary (top) and metastasized (bottom) h-HCCs circled by dashed blue lines (scalebars: 50 μm). (G) The rate of human hepatocyte repopulation in control and jet-lagged mice. (H) The h-HCC risk among TIRF mice generated by each human donor. (I) Serum levels of glucose, insulin, ALT, TNFα, and IL-6 in control mice with healthy livers (Ctrl h-Liver) and jet-lagged and h-HCC-free mice with NASH and/or cirrhosis (CJ h-NASH/Cirrhosis). Student's *t* test, ANCOVA, and chi-square test, $^{**}p < 0.01$, $^{***}p < 0.001$ (±SEM). ALT, alanine aminotransferase; h-, humanized-; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; TIRF mice, transgene-free *Il2rg*$^{-/-}$;*Rag2*$^{-/-}$;*Fah*$^{-/-}$ mice.

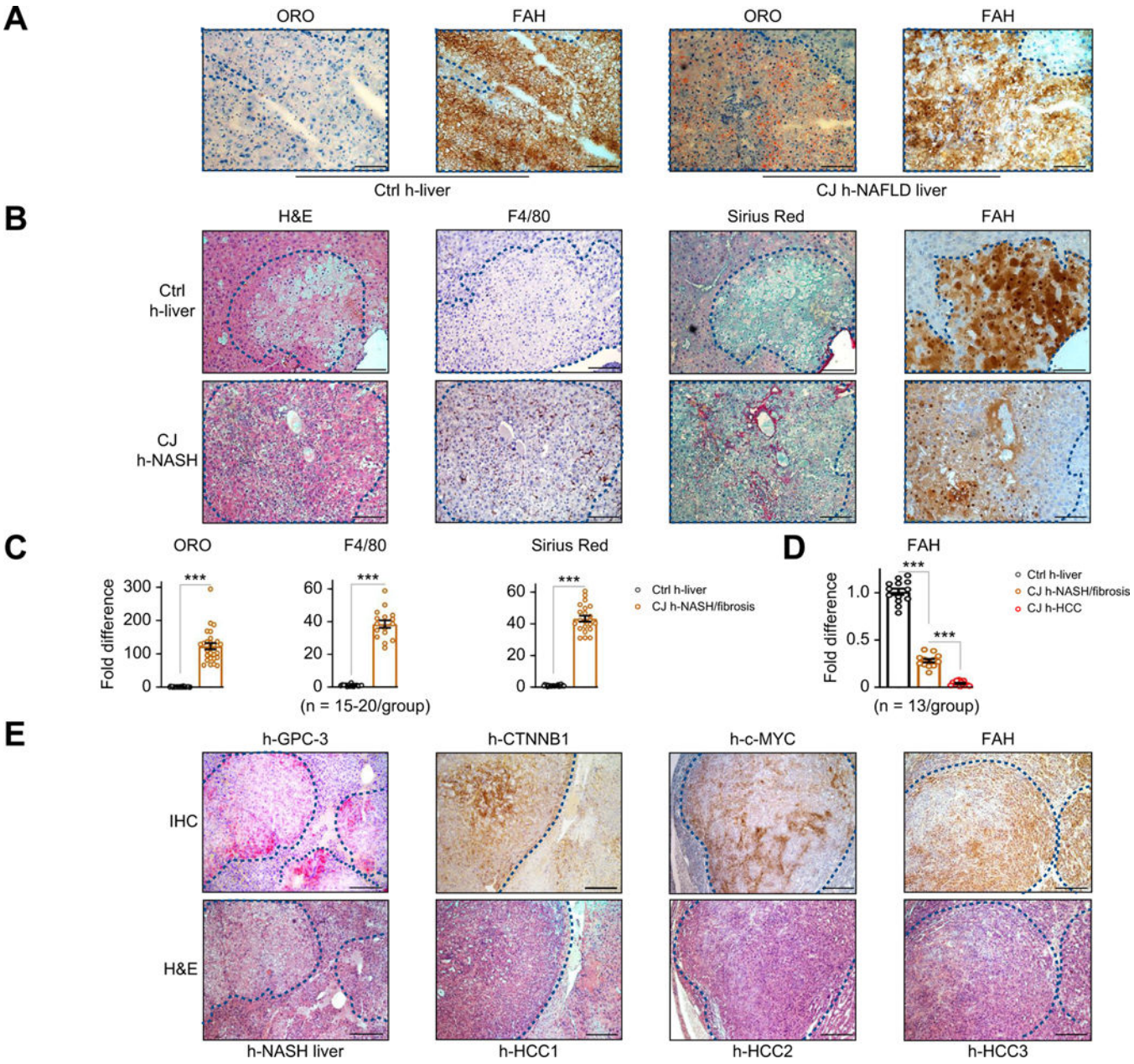



**Fig. 3. Circadian dysfunction Induces NAFLD-related h-HCCs.**

(A) ORO and FAH staining of consecutive cryostat sections show a healthy humanized liver (Ctrl h-liver) and a jet lag induced humanized NAFLD liver (CJ h-NAFLD). Note: only the FAH-positive humanized liver region shows fat accumulation. (B) H&E-, Sirius Red-, FAH-, and F4/80-stained consecutive sections of a Ctrl h-liver (top panel) and a jet lag induced humanized NASH liver (CJ h-NASH, lower panel). (C-D) The summary of ORO, F4/80, and Sirius Red (C) and FAH (D) signals in Ctrl h-liver, CJ h-NASH, and CJ h-HCC sections (10X magnification, Image J quantification, Student's *t* test, *$p$ <0.05). Signals in Ctrl h-Liver as arbitrary unit 1 (***$p$ <0.001, ±SEM). (E) Representative h-GPC-3, h-CTNNB1, h-c-MYC, and FAH IHC-stained (top panel) and H&E-stained (lower panel) consecutive sections of CJ h-NASH and CJ h-HCCs, circled by dashed blue lines (Scalebars: 50 μm in

A and B, and 100 μm in E). CJ, chronically jet-lagged; Ctrl, maintained in stable 24-hour LD cycles; DEGs, differentially expressed genes; h-, humanized-; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ORO, Oil Red O.

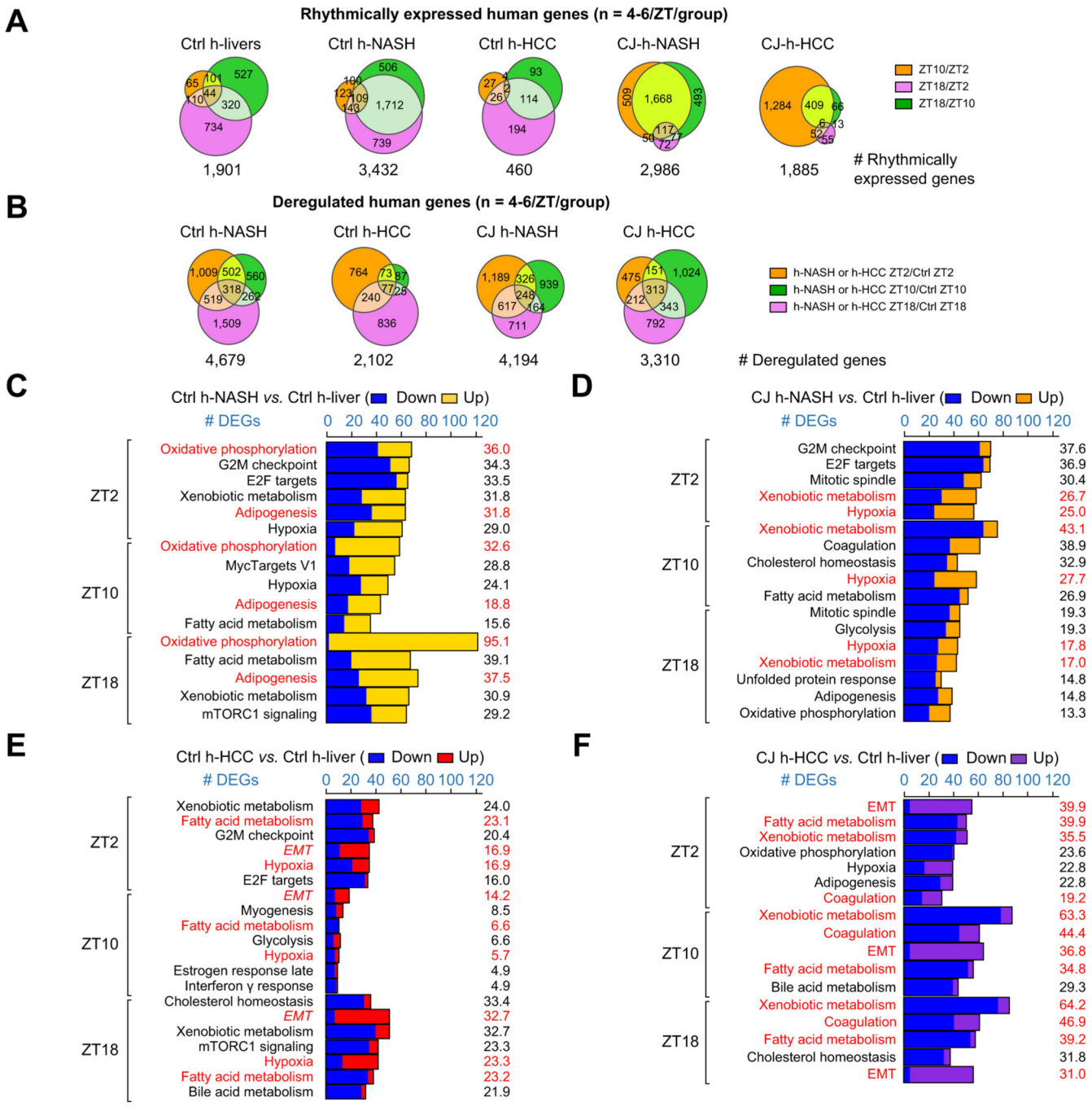



**Fig. 4. Chronic jet lag activates hallmarks of cancer in humanized livers and HCCs.**

(A) Venn diagrams show circadian expression of human genes in healthy h-livers and control and jet-lagged h-NASH and h-HCCs, defined by comparing DEGs between ZT10 and 2, ZT18 and 2, and ZT18 and 10 within each group. (B) The distribution of deregulated human genes at ZT2, 10, and 18 in control and jet-lagged h-NASH and h-HCCs, defined by comparing human genes expressed in h-NASH and h-HCCs to those in control healthy h-livers at same ZTs. (C–F) Top five deregulated human gene pathways in control and jet-lagged h-NASH and h-HCCs at ZT2, 10, and 18 defined by over-representation analysis

(red: among top five deregulated pathways at all ZTs studied). The length of bar indicates the total number of DEGs. The blue colour in bars: the numbers of downregulated genes. The yellow (C), orange (D), red (E), and purple (F) colour in bars: the numbers of upregulated genes. The number right next to each bar: -log10 *p* value of the pathway (**p* <0.05). CJ, chronically jet-lagged; Ctrl, under stably entrained condition; DEGs, differentially expressed genes; EMT, epithelial to mesenchymal transition; h-, humanized-; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; ZT, zeitgeber time. Venn Diagram and Over Representation Analysis (ORA, **p* <0.05).

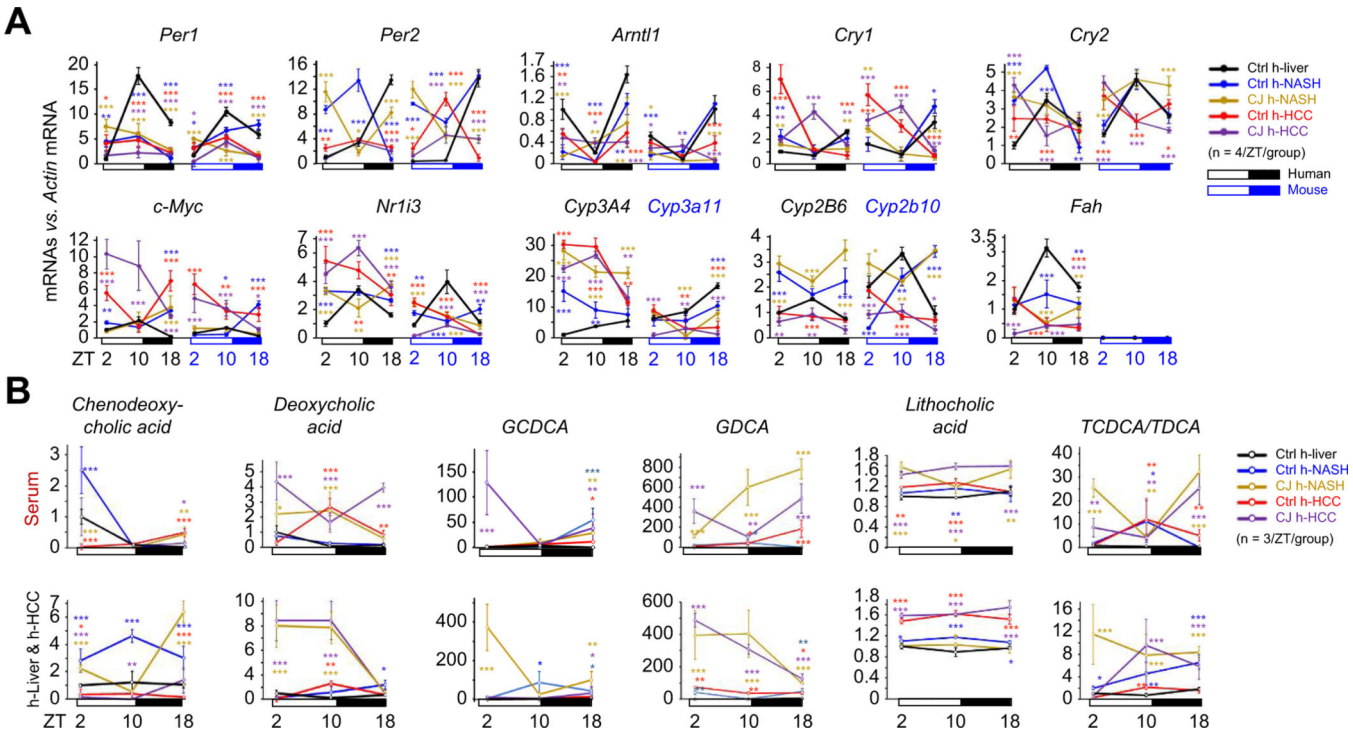



**Fig. 5. Jet lag disrupts the liver clock and deregulates serum and hepatic biomarkers.**

(A) Reverse-transcription PCR study of human (on black boxes) and mouse (on blue boxes) gene expression in the same h-livers or h-HCCs. Signals of each human gene expression in control h-livers at ZT2 are arbitrary unit 1. (B) Circadian profiles of representative BAs in serum (upper panel) and h-livers or h-HCCs (lower panel) (Ctrl h-Liver: healthy humanized livers isoalted under entrained condition; Ctrl h-NASH: mice with h-NASH but h-HCC-free under the entrained condition. CJ h-NASH: jet-lagged mice with h-NASH but h-HCC-free). Signals of BAs detected in serum and h-livers of control healthy mice at ZT2 are arbitrary unit 1. Student's *t* test and ANCOVA, **p* <0.05, ***p* <0.01; ****p* <0.001 (±SEM). BAs, bile acids; CJ, chronically jet-lagged; Ctrl, under entrained condition; h-, humanized-; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; ZT, zeitgeber time.

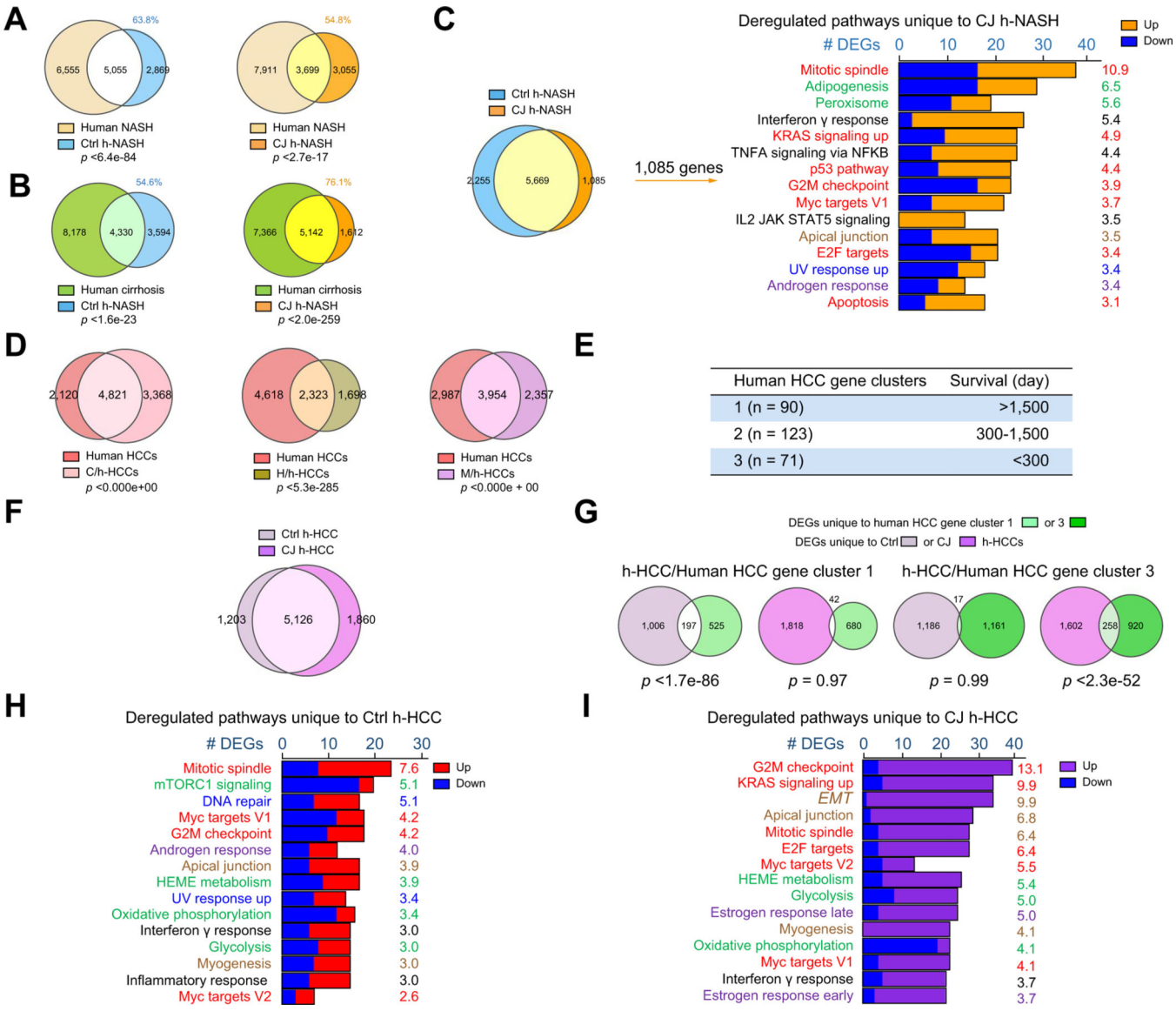



**Fig. 6. Humanized HCCs share the same molecular mechanism with human HCCs.**

(A-B) Venn diagram analysis of similarities of control and jet-lagged h-NASH transcriptomes with human NASH (A) and cirrhosis (B) transcriptomes. (C) Venn diagram shows shared and unique DEGs in control and jet-lagged h-NASH (left) and the top 15 jet lag induced deregulated pathways in NASH, defined by over-representation analysis of the 1,085 DEGs specific to CJ h-NASH (right). (D) Venn diagrams show the similarities between the human HCC transcriptome with the combined h-HCC transcriptome (C/h-HCC: derived from both human and mouse DEGs in all h-HCCs studied), the tumour-specific transcriptome (H/h-HCC: derived from human DEGs in all h-HCCs studied), and the tumour stroma-specific transcriptome (M/hHCC: derived from mouse DEGs in all h-HCCs studied). (E) Three human HCC gene clusters associated with differences in patient survival. (F) Venn diagrams show shared and unique DEGs in control and jet-lagged h-HCCs. (G) Venn diagrams comparing the similarities of control and jet-lagged h-HCC-specific

gene signatures with those unique to human HCC gene cluster 1 (best survival, left) and cluster 3 (worst prognosis, right). (H–I) The top 15 pathways unique to control (H) and jet-lagged (I) h-HCC transcriptomes (Red: cell proliferation, death, and oncogenic signalling. Blue: damage responses and repair. Black: immune responses. Green: metabolism and biosynthesis. Brown: epithelium-stroma interaction. Purple: sex disparity). Venn Diagram/phyper (*$p$ <0.05) and Over Representation (ORA,*$p$ <0.05) analyses. CJ, chronically jet-lagged; Ctrl, under entrained condition; DEGs, differentially expressed genes; h-, humanized-; HCC, hepatocellular carcinoma; NASH, nonalcoholic steatohepatitis.

**Table 1.**

Chronic circadian dysfunction increases the risk of humanized HCC.

| | | | Survival (week) | | Cause of death | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group** | **Donor** | **#Mice** | **Max.** | **Average** | **HCC** | **Cirrhosis/ jaundice** | **Kidney failure** | **Mice with h-HCCs** | **Total # h-HCC** | **h-HCC/ mouse** | **1st h-HCC detected (week)** | **Average age h-HCC found (week)** | ***p1*** | ***p2*** |
| Ctrl | M | 14 | 71+ | 58.2 | 9 (37.5%) | 1 (8%)[a] | 0[b] | 3 (21.4%) | 16 | 0.67 | 41 | 54 | | |
| | F | 10 | 71+ | 58 | | | | 2 (20%) | | | | | | |
| CJ | M | 14 | 60 | 38.6 | 19 (79%) | 2 (12.5%)[a] | 1 (4.2 %)[b] | 10 (71%) | 78 | 3.25 | 27 | 45 | <0.001 | <0.001 |
| | F | 10 | 60 | 38.07 | | | | 7 (70%) | | | | | | |
| CJ/CA | M | 14 | 60 | 33.5 | 15 (62.5) | 4 (16.7%)[a] | 2 (8.3%)[b] | 8 (57.1%) | 58 | 2.42 | 20 | 40 | <0.001 | =0.002 |
| | F | 10 | 60 | 34 | | | | 6 (60%) | | | | | | |

Ctrl, control mice; CJ, chronically jet-lagged mice; CJ/CA, mice chronically jet-lagged-and-cholic acid-fed; h-HCC, humanized HCC.

*p*1: The survival incidence in the CJ or CJ/CA group vs. that in controls, Kaplan-Meier statistics (**p* <0.05).

*p*2: The h-HCC incidence in the CJ or CJ/CA group vs. that in controls, Chi-Square Test (**p* <0.05).

[a]Severe cirrhosis/jaundice without HCCs.

[b]Severe hydronephrosis without HCC (All mice displayed certain degrees of hydronephrosis).

*Mice found dead with unknown causes due to severe tissue necrosis: 1 in Ctrl, 2 in CJ, and 3 in CJ/CA.