The circadian clock components BMAL1 and REV-ERBα regulate flavivirus replication

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The circadian clock regulates immune responses to microbes and affects pathogen replication, but the underlying molecular mechanisms are not well understood. Here we demonstrate that the circadian components BMAL1 and REV-ERBα influence several steps in the hepatitis C virus (HCV) life cycle, including particle entry into hepatocytes and RNA genome replication. Genetic knock out of Bmal1 and over-expression or activation of REV-ERB with synthetic agonists inhibits the replication of HCV and the related flaviruses dengue and Zika via perturbation of lipid signaling pathways. This study highlights a role for the circadian clock component REV-ERBα in regulating flavivirus replication.
The cell-autonomous circadian clock coordinates the network of physiological processes that define the daily rhythms of cell proliferation, metabolism and inflammation. Clock signalling pathways are primarily controlled by the transcription activators BMAL1 and CLOCK. The nuclear hormone receptors REV-ERβα and REV-ERββ are BMAL1-regulated clock components that provide a feedback loop that controls the expression of metabolic genes in a circadian and tissue-dependent manner. Host innate and adaptive immune response are now recognised to be circadian regulated and to influence susceptibility to infectious agents and response to vaccines. Pariollaud et al. recently reported a homeostatic role for REV-ERβα in regulating pulmonary inflammation, coupling the core clock to innate immunity. As obligate intracellular parasites viruses require host cell machineries and metabolites to replicate their viral genome and to assemble progeny virions. Recent studies reporting increased replication of herpes, influenza, respiratory syncytial virus and parainfluenza type 3 in Bmal1 knock-out mice suggest a role for circadian pathways to define host susceptibility to virus infection, however, the molecular mechanisms are not well understood. The recent availability of synthetic REV-ERβ ligands that modulate circadian pathways in vivo provide tools to study the role of REV-ERβ in viral replication and open exciting therapeutic opportunities for treating infectious disease.

The Flaviviridae family of positive-strand RNA viruses are major causes of morbidity and mortality and include the human pathogens: hepatitis C (HCV), dengue (DENV) and Zika (ZIKV) viruses. DENV infects around 390 million people per year, and the recently emerged ZIKV has been estimated to infect 750,000 individuals annually since 2015. To date, no anti-viral treatments are available for either virus. In contrast, the recent development of direct acting anti-viral agents (DAAs) against HCV infection has revolutionised treatment options. However, given the high cost and limited availability of these drugs, significant numbers of chronic HCV-infected individuals remain at risk to develop progressive liver disease and hepatocellular carcinoma. Despite differences in their transmission and pathogenesis, all of these viruses replicate in the cytoplasm and subvert lipid homeostatic pathways to induce organelle-like membrane structures that support viral replication.

The liver maintains an organism’s metabolic homeostasis and REV-ERβαs plays a key role in regulating bile acid and fatty acid biosynthesis. As HCV replicates solely in hepatocytes within the liver and there are excellent in vitro model systems available to study its replication, we investigated the role of circadian clock components in the HCV life cycle. We show a circadian cycling of HCV entry into hepatocytes that is defined via BMAL1 regulation of entry receptors CD81 and claudin-1. Furthermore, we show that REV-ERβα overexpression or activation with synthetic agonists inhibits HCV, DENV and ZIKV RNA replication, highlighting a new role for REV-ERβα to restrict RNA virus replication.

**Results**

**HCV infection is circadian regulated.** The human hepatocyte-derived cell line Huh-7 provides a well-characterised in vitro model to study the HCV life cycle and virus–hepatocyte interactions. Several approaches have been reported to synchronise the circadian clock in cell culture and we compared protocols that used dexamethasone, serum shock or temperature fluctuation to synchronise Huh-7 cells. Serum shocking the cells was the optimal protocol to coordinate the cycling of Bmal1 and Rev-erβα mRNA transcripts for 48 h (Fig. 1a), with the amplitude decreasing thereafter. Viral entry into a host cell represents the first step in the infectious life cycle and is mediated via specific interactions between virus proteins and cellular receptors that define particle internalisation pathways. Lentiviruses can incorporate exogenous viral encoded glycoproteins and the resulting pseudoparticles (pp) undergo a single cycle of infection that enable the study of receptor-specific internalisation pathways. Synchronised Huh-7 cells at different circadian times (CTs) were inoculated with HCV pp for 1 h, unbound virus was removed by washing and infection was measured after 24 h (Fig. 1b). HCV pp entry was maximal at CT8 and CT32 (Fig. 1c). Delivering the lentiviral DNA directly into cells showed that serum-induced circadian synchronisation had no effect on reporter activity per se suggesting an HCV glycoprotein receptor-dependent pathway (Supplementary Figure 1A). Infecting synchronised cells at CT0 or CT8 with pseudotyped viruses expressing HCV or vesicular stomatitis virus (VSV) glycoproteins confirmed that CT8 cells support greater levels of HCV pp infection that was not apparent for VSV pp infection (Supplementary Figure 1B). The infectious HCV cell culture (HCV cc) system recapitulates the complete viral life cycle and we evaluated the infectivity of two well-characterised HCV strains, J6/JFH-1 and SA13/JFH-1, to infect Huh-7 cells at defined CTs. Infection was quantified by counting viral antigen NS5A expressing cells and we observed a significant increase in infected cell numbers when the virus was inoculated at CT8 (Fig. 1d), suggesting that circadian pathways regulate HCV uptake into the liver.

HCV entry into hepatocytes is regulated by four essential host factors: CD81, scavenger receptor BI (SR-BI), claudin-1 and occludin. We investigated whether any of the viral receptors showed circadian expression and demonstrated a rhythmic pattern of CD81, claudin-1 and occludin mRNA levels in synchronised Huh-7 cells (Fig. 1e), with the peak of viral receptor transcripts coinciding with peak Bmal1 mRNA levels and particle uptake. To establish a link between BMAL1 and receptor expression, we transiently silenced Bmal1 in Huh-7 cells and observed a significant reduction in CD81 and claudin-1 transcripts (Supplementary Figure 2). CRISPR knockout (KO) of Bmal1 in Huh-7 cells confirmed a significant reduction in CD81 and claudin-1 mRNA and protein levels, with no detectable change in occludin (Fig. 1f). Importantly, these Bmal1 KO cells were resistant to HCV pp and HCV cc infection compared with wild-type (WT) cells (Fig. 1g). To ascertain whether BMAL1 regulates DENV or ZIKV infection, we infected the KO Huh-7 cells and observed a significant reduction in the replication of both viruses in Bmal1 KO cells compared with WT (Fig. 1h). As the mechanism of particle entry for these viruses into hepatocytes is poorly defined and the tools to study viral uptake are not well developed, we selected to study the role of BMAL1 in HCV entry. In summary, these data support a role for BMAL1 to regulate HCV, DENV and ZIKV infection.

Pharmacological activation of REV-ERβα inhibits HCV entry. Bmal1 is negatively regulated at the transcriptional level by REV-ERβαs, haem-binding transcriptional repressors. The development of synthetic agonists that activate REV-ERβα and modulate circadian pathways in vivo prompted us to investigate their ability to regulate HCV infection. REV-ERβα agonists, SR9009 and GSK2667, reduced BMAL1 promoter activity in a dose-dependent manner (Supplementary Figure 3) and we confirmed a reduction in endogenous mRNA levels and protein expression (Figs. 2a, b), with no detectable cytotoxicity or effect on hepatocellular viability (Supplementary Figure 3). To exclude the possibility of global transcriptional repression by these agonists, we assessed the expression level of nine housekeeping genes in treated Huh-7 cells and found no significant changes in their expression.
mRNA levels (Supplementary Figure 4). REV-ERB agonists treated Huh-7 cells showed a significant reduction in CD81 expression assessed by western blotting with negligible effects on claudin-1 or occludin expression (Fig. 2b). As measuring protein expression by western blotting is semiquantitative and partly defined by affinity and avidity of the antibodies being used, we selected a non-biased proteomic approach to assess the effect of SR9009 on viral receptor expression. We observed a significant reduction in CD81 and claudin-1 expression with an increase in occludin levels and no significant change in SR-BI (Fig. 2c).

Importantly, both REV-ERB agonists inhibited HCVpp entry in a dose-dependent manner (Fig. 2d). To evaluate the activity of these agonists against a wider spectrum of HCV strains, we utilised lentiviral pseudotypes expressing genetically diverse envelope glycoproteins and demonstrated inhibition of all strains tested (Fig. 2e). In summary, these data highlight a role for REV-ERB to regulate CD81 and claudin-1 protein expression and define circadian gating of HCV entry.

**REV-ERB limits HCV RNA replication.** HCV can establish a persistent infection and sub-genomic copies of the viral RNA can replicate autonomously. These replicon systems have been widely used as pre-clinical models for anti-viral drug discovery. Short hairpin RNA silencing Rev-erbα in Huh-7 cells stably supporting HCV-luciferase replicon increased viral replication (Figs. 3a, b),
REV-ERB regulates miR-122. HCV RNA binds the liver-specific microRNA miR-122 and this complex has been reported to support the replication of the viral RNA. As REV-ERB regulates miR-122 and both primary (pri)-miR-122 transcription and miR-122 target genes show circadian patterns of expression, we were interested to investigate the miR-122 dependency of REV-ERB regulation of HCV replication. Synchronised Huh-7 cells show a circadian pattern of pri-miR-122 expression (Fig. 4a) and REV-ERB agonists reduced pri-miR-122 levels (Fig. 4b). Co-transfection of a miR-122 antagonist along with HCV RNA into Huh-7 cells reduced virus replication as previously reported and both REV-ERB agonists showed reduced anti-viral activity (Fig. 4c), suggesting a role for REV-ERB regulation of miR-122 in restricting HCV replication. To investigate further, we evaluated the sensitivity of a HCV variant encoding a mutated miR-122 binding motif that recognised miR-15a/b (m15) to REV-ERB agonists. Both SR9009 and GSK2667 inhibited m15 replication with comparable efficiency as WT virus (Fig. 4d), demonstrating that REV-ERB agonists inhibit HCV replication independent of miR-122 binding to the viral RNA. As miR-122 regulates cholesterol and lipid metabolism, this most likely contributes to the anti-viral activity of REV-ERB agonists to limit HCV replication.

REV-ERBα regulates pathways essential for HCV, DENV and Zika. REV-ERBα regulates lipid and cholesterol metabolism in the murine liver. As lipids play a role in the genesis and maintenance of membranous vesicles that are essential for RNA virus replication and particle assembly, we investigated the effect of the REV-ERB agonists on de novo lipogenic pathways in human hepatocytes. As SR9009 showed the highest anti-viral activity and has been extensively studied in vivo, we performed a whole-genome microarray on SR9009 (20 µM) treated Huh-7 cells. Differentially expressed genes from the microarray data were filtered through the following criteria: Log2 fold change magnitude > 0.5, a p-value < 0.05, which generated a list comprising 4033 upregulated genes and 3660 downregulated genes (most highly regulated genes are listed in Supplementary Data 1). KEGG pathway analysis of the differentially expressed genes identified an enrichment of metabolic pathways involved in lipogenesis and cholesterol/bile acid metabolism (Figs. 5a, b). We demonstrated that SR9009 reduced stearoyl-CoA-desaturase (SCD) promoter activity, mRNA transcript levels and protein expression assessed by western blotting and proteomics analysis (Fig. 5c). Furthermore, SR9009-treated Huh-7 cells showed a significant reduction in unsaturated fatty acid levels (16:1, 16:0—ctrl 0.42v SR9009 0.38, p = 0.0018; 16:1 + 18:1—ctrl 20.41v SR9009 18.98, p = 0.0001; 16:1 + 18:1—16:0—ctrl 0.77v SR9009 0.72, p = 0.0065), a phenotype consistent with reduced SCD expression and activity. Trump et al. reported that REV-ERB agonists activate the nuclear receptor LXRα in human monoocyte THP1 cells. As LXR regulates metabolic pathways that overlap with REV-ERB, we evaluated the ability of SR9009 to activate LXR and demonstrated a negligible effect on known LXR target gene transcription in human hepatocytes (Supplementary Figure 6). Analysis of published ChIP-seq data on REV-ERB chromatin occupancy in murine liver showed evidence of binding the SCD1 promoter (Fig. 5d). Overexpressing REV-ERBα in Huh-7 cells reduced SCD expression in a dose-dependent manner (Fig. 5e), consistent with our earlier data showing that endogenous REV-ERBα levels are low in Huh-7 cells (Fig. 3c). Cumulatively, these data show for the first time a role for REV-ERBα to regulate SCD in human hepatocytes.

As SCD is rate limiting for HCV infection, we investigated the contribution of this pathway to REV-ERB regulation of HCV replication. Oleic acid is the final product of SCD and can restore de novo lipogenesis pathways in cells lacking SCD.
Supplementing the culture media with oleic acid had a negligible effect on HCV replication (Supplementary Figure 7) but reduced the anti-viral activity of SR9009 and control SCD inhibitor (Fig. 5f). Furthermore, CRISPR knock-down (KD) of SCD in HCV replicon cells inhibits viral replication and reduced the anti-viral activity of SR9009 and SCDi (Fig. 5g), demonstrating that REV-ERB perturbation of SCD expression contributes to its anti-viral activity.

As the replication of many flaviviruses is dependent on fatty acid biosynthesis33, we investigated a role for REV-ERB in the replication of DENV and ZIKV. Transient expression of REV-ERBα in Huh-7 cells using a dose of plasmid previously

**Fig. 2** REV-ERB agonists inhibit hepatitis C virus (HCV) entry. a REV-ERB agonists inhibit BMAL1 transcription. Huh-7 cells were treated with REV-ERB agonists SR9009 or GSK2667 (20 µM) for 24 h and Bmal1 mRNA levels quantified by qRT-PCR, respectively (mean ± S.E.M., n = 7, Kruskal-Wallis ANOVA with Dunn’s test). b, c REV-ERB agonists modulate HCV receptor expression. Huh-7 cells were treated with the REV-ERB agonists SR9009 or GSK2667 (20 µM) for 24 h and the cells lysed, total protein measured and assessed for CD81, claudin-1 and occludin expression together with the housekeeping GAPDH by western blotting or mass spectrometric analysis (mean ± S.E.M., n = 3, Mann-Whitney test). d REV-ERB agonists inhibit HCV entry. Huh-7 cells were treated with increasing dose of REV-ERB agonists SR9009 or GSK2667 for 24 h, inoculated with HCVpp and infection assessed 24 h later (mean ± S.E.M., n = 5). e REV-ERB agonists inhibit HCVpp bearing patient-derived glycoproteins. Huh-7 cells were treated with the REV-ERB agonists SR9009 or GSK2667 (20 µM) for 24 h, infected with HCVpp bearing patient-derived envelope glycoproteins and infection assessed 24 h later. In all cases, data are expressed relative to untreated (Ctrl) cells. (Mean ± S.E.M., n = 3, Kruskal-Wallis ANOVA)
Fig. 3 REV-ERBα inhibits hepatitis C virus (HCV) RNA replication. a Silencing Rev-erbα increases HCV replication. Huh-7 cells supporting a HCV JFH-1-LUC replicon were transduced with lentivirus encoding shRev-erbα or control and silencing confirmed by measuring Rev-erbα mRNA and protein expression levels (mean ± S.E.M., n = 4, Mann-Whitney test). Densitometric analysis quantified REV-ERB in individual samples and was normalised to its own GAPDH loading control. HCV replication-dependent reporter activity was measured and expressed relative to control (shCtrl) cells (mean ± S.E.M., n = 6, Mann–Whitney test). b Anti-viral activity of SR9009 agonist is dependent on REV-ERB expression levels. shRev-eRbα and Ctrl HCV JFH-1 replicon cells described in (a) were treated with REV-ERB agonist SR9009 for 24 h, viral replication measured and the concentration of agonist required to inhibit viral replication by 50% defined (IC50) (mean ± S.E.M., n = 3). c REV-ERBα overexpression inhibits HCV RNA replication. Huh-7 cells stably supporting a HCV JFH-1-LUC replicon were transfected with empty plasmid or vector expressing REV-ERBα and Ctrl 48 h later protein expression assessed by western blotting and viral replication measured (mean ± S.E.M., n = 4, Mann–Whitney statistical test). Data are plotted relative to Ctrl untreated cells. d REV-ERB agonists cure HCV-infected cells. HCVcc SA13/JFH-1 infected Huh-7 cells were treated with increasing concentrations of REV-ERB agonists for 24 h and viral RNA or NS5A-expressing cells quantified and data expressed relative to Ctrl untreated cells. The experiment was performed in the presence of a neutralising anti-CD81 antibody to limit secondary rounds of infection (mean ± S.E.M., n = 3). e REV-ERB ligands inhibit the replication of diverse HCV genotypes. Huh-7 cells transiently supporting HCV sub-genomic replicons representing genotypes 1–3 were treated with the REV-ERB agonists SR9009 or GSK2667 and replication assessed 24 h later. The dose of agonist required to inhibit HCV RNA replication by 50% (IC50) was determined for all viral genotypes (mean ± S.E.M., n = 3)
shown to inhibit HCV replication significantly reduced DENV and ZIKV replication (Fig. 6a). Treating Huh-7 cells with REV-ERB agonist SR9009 reduced DENV or ZIKV infection, as assessed by measuring secreted levels of viral RNA and infectious particles (Fig. 6b). Furthermore, CRISPR KD of SCD in DENV or ZIKV-infected Huh-7 cells reduced viral replication and the anti-viral activity of SR9009 (Fig. 6c), highlighting SCD as a common pathway for REV-ERB to regulate this family of viruses.

**Discussion**

In this study, we examined the interplay between the circadian regulatory pathways and the replicative life cycle of HCV, DENV and ZIKV. Our results provide the first mechanistic evidence for circadian gating of HCV entry into hepatocytes via the regulation of entry receptors CD81 and claudin-1, providing a potential mechanism for our earlier observation reporting a time-of-day dependence in HCV re-infection kinetics following liver
transplantation. We discovered a new role for REV-ERB to regulate HCV, DENV and ZIKV RNA replication by repressing additional pathways contributing to its anti-viral activity. However, our data provide the first evidence that pharmacological modulation of circadian pathways can inhibit viral replication. Targeting host pathways that are essential to virus replication can provide therapies that generate limited resistance and such chronotherapies may work synergistically with DAAs.
Our data showing rhythmic expression of CD81, claudin-1 and occludin transcripts in Huh-7 cells allows for the potential to regulate HCV entry into hepatocytes. Analysis of published Chipseq datasets from murine liver35,36 reveals that BMAL1 binding to the viral RNA, and the infectivity titre of secreted virus was quantified and the data expressed relative to untreated control cells, where the mean titre of virus secreted from untreated cells was 1.6 × 10^5 pfu/ml. Huh-7 cells were infected with ZIKV MP1751 at an MOI of 0.1 and treated with SR9009 for 48 h. Extracellular RNA and infectious titre was quantified and the data expressed relative to untreated cells, where the mean titre of virus secreted from untreated cells was 1.6 × 10^5 pfu/ml. Huh-7 cells were infected with ZIKV MP1751 at an MOI of 0.1 and treated with SR9009 for 48 h. Extracellular RNA and infectious titre was quantified and the data expressed relative to untreated cells, where the mean titre of virus secreted from untreated cells was 2.9 × 10^5 pfu/ml. A role for SCD in REV-ERB agonist inhibition of DEN and ZIKV replication. DENV replicon cells or ZIKV-Nanoluc infected cells were transfected with CRISPRs targeting exons 2 and 3 of SCD or a scrambled guide RNA and 24 h later treated with SR9009. SCD expression was measured by western blotting and the dose of REV-ERB agonist required to inhibit DENV or ZIKV replication by 50% (IC_{50}) in WT or KD cells determined (mean ± S.E.M., n = 6, Mann-Whitney statistical test).

Recent studies reporting increased replication of herpes, influenza, respiratory syncytial virus and parainfluenza type 3 viruses in Bmal1-/— mice suggest that BMAL1 negatively regulates viral infection9,19. However, these studies did not address the underlying mechanism. Our observation that HCV, DENV and ZIKV show reduced replication in Bmal1 KO hepatocytes highlights the diverse roles of BMAL1 in the life cycle of different virus families that requires further investigation. In vivo small animal models for studying HCV, DENV or ZIKV replication frequently use immunocompromised mice11-13 that limit studies to evaluate the immunomodulatory role of BMAL1 or REV-ERB in the viral life cycle.

Since virus infection, replication and particle assembly are dependent on cellular membranes, we examined the lipid composition of S9009-treated Huh-7 cells and observed changes in both neutral (particularly triglycerides) and phospholipids. Notably, the lipidomic analysis highlighted selective changes in lipid molecular species, rather than a generalised change suggesting selective effects on lipid turnover. Of particular note was the 60% reduction in total cellular phosphatidic acid concentration, reflected almost exclusively in the mono- and di-unsaturated species (Supplementary Figure 8). This reduction is an unexpected consequence of SCD downregulation reducing monounsaturated fatty acid generation and is likely to induce a profound effect on cellular membranes. Phosphatidic acid is unique in that its small, highly charged head group is close to its glycerol backbone, allowing it to induce a high membrane curvature, which may play a role in viral RNA replication and particle assembly. In contrast, the unsaturated phosphatidic acids are more loosely packed in the...
membrane and induce less curvature. Consequently, we propose that the selective loss of mono- and di-unsaturated phosphatidic acid species will have a major effect on membrane structure and intracellular viral function.

Ultrastructural and functional studies show a role for SCD in the genesis of viral replication compartments or ‘replication factories’ in HCV, DENV and ZIKV but also West Nile, human immunodeficiency virus, respiratory syncytial virus and other respiratory viruses, extending the significance of our observations to a wider spectrum of human pathogens. These viral replication factories serve multiple purposes: (1) spatial separation of different steps in the viral replication cycle, namely, RNA translation, replication and packaging of viral genomes into particles to prevent interference; (2) enabling a high local concentration of viral replicase complex components and metabolites to maximise RNA amplification and (3) protecting newly synthesised viral RNA from innate immune surveillance. To date, our understanding of the role circadian factors play in viral replication at a cellular level is limited. Our findings show new pathways for the circadian network to impact the peregrine complex and metabolites such as nucleotides to maximise RNA amplification and protecting newly synthesised viral RNA from innate immune surveillance. To date, our understanding of the role circadian factors play in viral replication at a cellular level is limited. Our findings show new pathways for the circadian network to impact the peregrine complex and metabolites such as nucleotides to maximise RNA amplification and protecting newly synthesised viral RNA from innate immune surveillance. To date, our understanding of the role circadian factors play in viral replication at a cellular level is limited. Our findings show new pathways for the circadian network to impact the peregrine complex and metabolites such as nucleotides to maximise RNA amplification and protecting newly synthesised viral RNA from innate immune surveillance. To date, our understanding of the role circadian factors play in viral replication at a cellular level is limited. Our findings show new pathways for the circadian network to impact the peregrine complex and metabolites such as nucleotides to maximise RNA amplification and protecting newly synthesised viral RNA from innate immune surveillance.

**Methods**

**Cell culture.** The human hepatoma cell line HuH-7 (gift from C. Rice, Rockefeller University, NY) and HCV sub-genomic replicon line A2-Luc (gift from R. Thimme, University of Freiburg, Germany) were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/10% foetal bovine serum (FBS)/1% non-essential amino acids/1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). HuH-7 cells were synchronised by treating with culture medium containing 50% FBS for 1 h. BMAL1 KO HuH-7 clones were generated by transfecting a pool of three BMAL1 KO plasmids (Santa Cruz Biotechnology, UK) followed by fluorescence activated cell sorting (FACS) and clonal expansion. LLC-MK2 and AFRIMS kidney cells (ATCC, UK) were maintained in M199 medium/10% FBS/1% penicillin/streptomycin. Vero epithelial kidney cells (ATCC, UK) were maintained in DMEM/10% FBS/1% penicillin/streptomycin.

**Reagents and antibodies.** Following reagents were purchased from commercial suppliers: REV-ERBα expression plasmid (Origene, UK); SCD promoter-luc plasmid (GeneCopoeia, USA); 2-hydroxysterol (Calbiochem, USA); Daclatasvir and Sofosbuvir (Selleckchem, USA) and SCD inhibitor A939572 (BioVision, USA). 2-hydroxysterol was synthesised at University of Birmingham. All drugs were dissolved in dimethyl sulfoxide (DMSO) and their cytotoxicity determined by a Lactate dehydrogenase (LDH) assay (Promega, UK). The following primary antibodies were used: anti-NS5A 9E10 (1 µg/mL, C. Rice, Rockefeller University), anti-CD81 (1 µg/mL, 2.131), anti-CLAUDIN-1 (1 µg/mL Abcam, UK), anti-occludin (2 µg/mL, Invitrogen, UK), anti-BMAL1 (1 µg/mL, Abcam, UK), anti-REV-ERBα (1 µg/mL, Thermo Fisher Scientific, UK), anti-SCD (1 µg/mL, Abcam, UK) and anti-GAPDH (1 µg/mL, Cell Signaling, UK). Uncropped original western blots are shown in Supplementary Figure 9. Fluorescent Alexa Fluor 488-conjugated anti-mouse secondary antibodies were obtained from Invitrogen, UK.

The BMAL1 promoter was amplified from genomic DNA using forward primer: 5’-CCGCTCGAGGGGAGCAAGCGGAGGTCGACG-3’ and reverse primer: 5’-CCCAGTCTCGGAGGCGGCGGCGGCGCAGTCGACG-3’ and reverse primer: 5’-CCGCTCGAGGGGAGCAAGCGGAGGTCGACG-3’ and reverse primer: 5’-CCCAGTCTCGGAGGCGGCGGCGGCGCAGTCGACG-3’ and cloned into the pRL3 luciferase reporter vector (Promega, UK). Lenti-sh-REV-ERBα construct was a gift from Dr. B. Grimaldi, University of Genoa, Italy. To generate SCD KO cells, CRISPR guide RNA (gRNA) forward and reverse sequences were designed using CRISPR Finder (https://www.sanger.ac.uk/hgpt/ogre/find_crispr) with overhang sequences containing a BbsI restriction site. The gRNA sequences were annealed and cloned into BbsI-digested pSpCas9(BB)-2A-Puro (PX459) V2.0 DNA (Addgene plasmid #62898; deposited by Dr. Feng Zhang). The CRISPR gRNA plasmid products were sequenced to confirm successful cloning. SCD exon 2 gRNA

**Fig. 7** Model of circadian clock components regulating HCV, DENV and ZIKV replication. The circadian activator BMAL1 regulates HCV entry into hepatocytes through modulating viral receptors CD81 and claudin-1 expression. Activating REV-ERB with synthetic agonists or protein overexpression inhibits HCV, DENV or ZIKV RNA replication via modulating SCD and subsequent release of infectious particles.
sequences: exon 2 forward—5′-CAGGCGGCTTCTTACTCTGTTGA-3′ and exon 2 reverse 5′-AACCACTAACAGGTAAGAAAGGCC-3′. SCID exon 3 GRNA sequences: exon 2 forward—5′-CAGGCGGCCCGCCCGTGTGAA-3′ and exon 3 reverse 5′-AAACACTCTTAAACAAGCTGGTCG-3′. All primers used are detailed in Supplementary Table 1.

HCVcc and HCVpp infection. Plasmids encoding HCV SA13/FHJ and JFH-1 were transfected into the Huh-7.5 cells56,57. Huh-7.5 cells were infected with JFH-1-luc (genotype 2a) and S52-ΔIle luciferase (LUC) gene in place of DENV2 C, prM and E genes and flanked by artificial 5′ and 3′ RACE primers. Viral antigen-expressing cells were enumerated using a fluorodeoxyuridine (FUDR). For ZIKV, the envelope primer set consisted of forward 5′-CTCTGCTACACTTGGGAGCC-3′ and probe 5′-GAGCTCCTGCTGTTATGCCC-3′. The mobile phase was constant at 200 µl/min. Accurate mass (with an error below 5 ppm) was acquired on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, USA). Source parameters for negative polarity were: capillary temperature 325 °C; source heater temperature 325 °C; sheath gas 10 AU; aux gas 5 AU; normalized collision energy 32.5. Full range of m/z 200–1000 were acquired at a target resolution of 240,000 (FWHM at m/z 400). Data were analysed using Lipid Analyst Data (2.0.0–2.0) software.

Proteomic analysis. Three biological replicates of control and SR9009-treated Huh-7 cells were lysed in RIPA buffer and peptides separated using NanoPAGE 4–12% bis–tris gels (Invitrogen). Peptides obtained from selected proteins (CD81, claudin-1, occludin, SB-Bl, occluding, SCID) in gel trypsin digestion (trypsin pH 8) were digested and the resulting peptides quantified using liquid chromatography–mass spectrometry (LC-MS/MS) methods. Peptides were desalted using an online C18 trap column (PepMap 100, 5 µm particle size, 300 µm i.d. × 5 mm, Thermo Fisher Scientific) and for data-dependent acquisition (DDA) and parallel reaction monitoring (PRM). DDA was used for discovery and library creation of peptides for the purpose of PRM-based quantitation. DDA method settings included: chromatographic peak width: 12 s, the full scan (MS1) resolution: 70,000, 40,000; AGC target: 3e5, maximum injection time (IT): 20 ms, scan range: 375–1500 m/z. The dd-MS2 conditions—resolution: 17,500. The AGC target conditions—1e5, maximum IT: 60 ms, loop count: 10; (i.e., Top 10), isolation width: 2.0 m/z, fixed first mass: 100.0 m/z, and the data-dependent (dd) settings—minimum AGC target: 2e3, intensity threshold: 3e4, charge exclusion: unassigned, 1, > 8, peptide match; preferred, dynamic exclusion: 20 s. Normalised collision energy (NCE) of 27 was used for fragmentation of peptides in a high-energy collision dissociation (HCD) cell. PRM method settings: global settings—user role: advanced; lock mass: best; chromatographic peak width: 20 s; full MS-SIM setting resolution: 17,500; AGC target: 2e5; maximum IT: 200 ms; MSX count: 1; isolation window: 1.6 m/z; normalised collision energy (NCE): 27; spectrum type: profile. The MS tune parameters: for data-dependent reactions: full scan m/z range: 350–2000 m/z; fragmentation: none, resolution: 70,000, polarity: positive, microscan: 1; AGC target: 1e5, maximum IT: 100; sheath gas flow: 0; aux gas flow: 4.5

Analysis of cellular triacylglycerol fatty acids and phospholipids. Total lipids from mock or SR9009 (20 µM)-treated Huh-7 cells were extracted using the Folch method56. The triacylglycerol fraction was separated using solid phase extraction columns and fatty acid methyl esters (FAMES) prepared by incubating with methanolic sulphuric acid at 80 °C for 2 h. Gas chromatography was performed using an Agilent 6890 GC (Agilent, UK) equipped with a programmed temperature split–splitless injector and a fused silica capillary column (50 m × 0.25 mm i.d., film thickness 0.25 µm). Helium was used as carrier gas (1 mL/min) and the oven temperature was programmed to start at 120 °C for 6 min and then ramp to 250 °C at a rate of 4 °C min−1. ChIP-seq datasets were obtained from the Gene Expression Omnibus where peak detection was performed using the HOMER tool. BedGraphs were visualised using the Integrative Genomics Viewer (IGV) tool65 overlaying the mouse genome (mm10).

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The Qiagen Viral RNA mini kit according to the manufacturer protocol. ZIKV strain MP1751 at an MOI of 0.1 for 90 min at RT, unbound virus was precipitated with 8% w/v PEG in NTE buffer. Huh-7.5 cells were infected with the infectious titre of infection (MOI) of 1 for 90 min at room temperature (RT). Unbound seed was inoculated into 24-well plates and 24 h later infected with DENV2 at a multiplicity of infection (MOI) of 0.1 for 90 min at RT, unbound virus was removed by washing and the cultures treated with SR9009 or control DMSO and incubated for 48 h. Supernatants were harvested and the infectious titre determined on LLC-MK2 cells. Viral RNA was extracted from the supernatant using the Qiagen Viral RNA mini kit according to the manufacturer’s protocol.

ZIKV strain MP1751 (PHE, UK) was propagated in Vero cells, concentrated by precipitation with 8% w/v PEG in NTE buffer. Huh-7.5 cells were infected with ZIKV strain MP1751 at an MOI of 0.1 for 90 min at RT, unbound virus was removed by washing and the cultures treated with SR9009 or control DMSO and incubated for 48 h. Supernatants were harvested and the infectious titre determined by plaque assay on Vero cells. Viral RNA was extracted from the supernatant using the Qiagen Viral RNA mini kit according to the manufacturer’s protocol. The cell membranes were then solubilized using a standard protocol to generate genomic DNA by PCR using the following primers: forward 5′-GGATTAAGCTGAGGGT-3′ and reverse 5′-TAGACCCATGGATTTCCCCA-3′. The DNA was transfected into Vero E6 cells (ATCC, CRL-81) by use of Lipofectamine 2000 as per the manufacturer’s instructions (Thermo Fisher Scientific). Viral RNA stocks were titrated in the A549/BVDV-Npro cell line54. Huh-7.5 cells infected with ZIKV-Nanoluc at an MOI of 0.1 and treated with SR9009 or DMSO vehicle alone for 48 h and Nluc activity was measured using the Nano-Glo kit (Promega).

Real-time reverse transcription PCR. Purified total RNA samples were tested for HCV RNA expression (Primer Design Ltd, UK) in a qRT-PCR as per the manufacturer’s guidelines. The primer and probe sequences were adapted from previously published methods36,38. For DENV, the NS5 primer set consisted of forward 5′-CAAGTTGCAAATCCGTCCT-3′, reverse 5′-GGCCGACATTGTTCTCTC-3′ and probe 5′-(6FAM) CCATTG AAATCTGGGAAAGAACTCCTAAMA3′ (5′). For ZIKV, the primer envelope set consisted of forward 5′-CAAGTCCAGACTGCTGTTGA-3′ and reverse 5′-GAGCCTCGTGGTTATGACC-3′ was used in a SYBR green qRT-PCR (Qiagen, UK).

DENV and ZIKV RNA was assayed by qRT-PCR on an Applied Biosystems 7500 real-time PCR system using the Verso 1-step RT-PCR kit with Thermoscript Taq (Applied Biosystems) as per the manufacturer’s guidelines. The primer and probe sequences were adapted from previously published methods36,38. For DENV, the NS5 primer set consisted of forward 5′-CAAGTTGCAAATCCGTCCT-3′, reverse 5′-GGCCGACATTGTTCTCTC-3′ and probe 5′-(6FAM) CCATTG AAATCTGGGAAAGAACTCCTAAMA3′ (5′). For ZIKV, the primer envelope set consisted of forward 5′-CAAGTCCAGACTGCTGTTGA-3′ and reverse 5′-GAGCCTCGTGGTTATGACC-3′ was used in a SYBR green qRT-PCR (Qiagen, UK).

HCV and DENV sub-genomic replicons. Plasmids encoding the HCV and DENV sub-genomic replicons were generated37,39. The HCV L-GDD con1 (genotype 1b), H77 and from patient-derived strains were engineered using a NS2B-NS3 cleavage site at the C-terminus enables cleavage from the rest of the polyprotein. In all, 2 µg of HCV or DENRNA was electroentrapped into 4 x 104 cells and allowed to recover for 48 h before treating with REV-ERB ligands. Huh-7 cells stably expressing HCV RNA were generated from N17 plasmid35. Cells were seeded in well cell plates, incubated with Viro-Glo (Promega, UK) and monitored in real-time at 30-min intervals over a 24 h period on a microplate reader (Clariostar, BMG Labtech, UK).

Microarray and ChIP-seq analyses. Microarray datasets for SR9009 treated Huh-7 cells (20 µM, 24 h treatment, n = 3) were obtained from Oxford Technology, UK. Differentially expressed genes were filtered using the following parameters: p-value < 0.05, log2FC > 0.5 or log2FC < −0.5, KEGG profiling30 was used to perform genome mapping and clusters were re-examined using significantly upregulated transcripts. Metabolic-related pathways were extracted and represented as bar charts with the colour of each bar indicative of its p-value. ChIP-seq datasets for murine REV-ERBα and REV-ERBβ were obtained from Gene Expression Omnibus where peak detection was performed using the HOMER tool. BedGraphs were visualised using the Integrative Genomics Viewer (IGV) tool65 overlaying the mouse genome (mm10).
0, sweep gas flow: 0, spray voltage: 2.3 kV, capillary temperature: 320 °C, S-lens RF level: 50. Data acquired using DDA were processed using the Mascot search engine against human Uniprot protein database. Mascot search results were uploaded to Skyline (version 4.1.0.18169, 64-bit, Seattle, USA) to create a reference library. PRM data were processed using Skyline to quantify peptides in different samples.

**Statistical analysis.** Statistical analyses were performed in Graph Pad Prism 7 (GraphPad, USA) using Mann–Whitney’s test or Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn’s test (for multiple comparisons), except where stated otherwise, with a p-value of <0.05 considered statistically significant (p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The metabolomics data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTRLS792. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD011721 and 10.6019/PXD011721, and can be accessed via ftp://PRIDE/2013022/PXD/765/va/ftp-peptidedata.org/. The microarray data from SRP009 treated Huh-7 cells have been deposited at NCBI GEO with the identifier GSE123748 and can be accessed via: ftp://GSE123748. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors upon request. Received: 7 May 2018 Accepted: 17 December 2018 Published online: 22 January 2019

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Author contributions
X.Z. designed study, conducted experiments and co-wrote manuscript; A.M. conducted experiments; M.H. conducted experiments; A.G.L. conducted analyses; A.Kumar conducted experiments; S.B.R. conducted experiments; C.L.D. conducted experiments; A.L. conducted experiments; S.R. provided reagents; K.P. conducted experiments; W.H.C. performed bio-informatic analyses; P.A.C.W. conducted experiments; R.B. synthesised compounds; X.Q. provided reagents; P.S. edited manuscript; T.B. provided reagents; D.R. edited manuscript; A.L. edited manuscript; P.B. provided statistical evaluation and co-wrote manuscript; M.W. co-wrote manuscript; A.Khol provided reagents and edited manuscript; C.L.I. provided reagents and edited manuscript; N.Z. provided reagents and edited manuscript; J.A.M. designed the study and wrote the manuscript.

Additional information
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