Initial Hepatitis C Virus Infection of Adult Hepatocytes Triggers a Temporally Structured Transcriptional Program Containing Diverse Pro- and Antiviral Elements

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ABSTRACT Transcriptional profiling provides global snapshots of virus-mediated cellular reprogramming, which can simultaneously encompass pro- and antiviral components. To determine early transcriptional signatures associated with hepatitis C virus (HCV) infection of authentic target cells, we performed *ex vivo* infections of adult primary human hepatocytes (PHHs) from seven donors. Longitudinal sampling identified minimal gene dysregulation at 6 h postinfection (hpi). In contrast, at 72 hpi, massive increases in the breadth and magnitude of HCV-induced gene dysregulation were apparent, affecting gene classes associated with diverse biological processes. Comparison with HCV-induced transcriptional dysregulation in Huh-7.5 cells identified limited overlap between the two systems. Of note, in PHHs, HCV infection initiated broad upregulation of canonical interferon (IFN)-mediated defense programs, limiting viral RNA replication and abrogating virion release. We further find that the constitutive expression of *IRF1* in PHHs maintains a steady-state antiviral program in the absence of infection, which can additionally reduce HCV RNA translation and replication. We also detected infection-induced downregulation of ~90 genes encoding components of the eIF2 translation initiation complex and ribosomal subunits in PHHs, consistent with a signature of translational shutoff. As HCV polyprotein translation occurs independently of the eIF2 complex, this process is likely proviral: only translation initiation of host transcripts is arrested. The combination of antiviral intrinsic and inducible immunity, balanced against proviral programs, including translational arrest, maintains HCV replication at a low level in PHHs. This may ultimately keep HCV under the radar of extrahepatocyte immune surveillance while initial infection is established, promoting tolerance, preventing clearance, and facilitating progression to chronicity.

IMPORTANCE Acute HCV infections are often asymptomatic and therefore frequently undiagnosed. We endeavored to recreate this understudied phase of HCV infection using explanted PHHs and monitored host responses to initial infection. We detected temporally distinct virus-induced perturbations in the transcriptional landscape, which were initially narrow but massively amplified in breadth and magnitude over time. At 72 hpi, we detected dysregulation of diverse gene programs, concurrently

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promoting both virus clearance and virus persistence. On the one hand, baseline expression of *IRF1* combined with infection-induced upregulation of IFN-mediated effector genes suppresses virus propagation. On the other, we detect transcriptional signatures of host translational inhibition, which likely reduces the processing of IFN-regulated gene transcripts and facilitates virus survival. Together, our data provide important insights into constitutive and virus-induced transcriptional programs in PHHs and identify simultaneous antagonistic dysregulation of pro- and antiviral programs, which may facilitate host tolerance and promote viral persistence.

KEYWORDS hepatitis C virus (HCV), RNA-seq, primary human hepatocytes, IFN regulatory factor 1 (*IRF1*), IFN signaling, eIF2 signaling, translational shutoff

Despite the development of effective antiviral therapies, hepatitis C virus (HCV) remains a global health burden and still chronically infects around 71 million people worldwide. HCV is a positive-stranded RNA virus of the *Flaviviridae* family with a tropism restricted to human hepatocytes (1). The development of cell culture systems has yielded insights into HCV-host interactions. For instance, lipid metabolic reprogramming favorable to the constitution of the viral replication organelle (2) and stress responses (3) were reported upon HCV infection. These dysregulations facilitate viral propagation but also the development of pathogenesis in chronic infection, including liver steatosis (4). Balanced against this, HCV infection elicits a classical innate immune response that obeys rules that are conserved across viruses (5). Virus infection is sensed via diverse pattern recognition receptors (PRRs), which trigger signaling cascades involving the nuclear translocation of interferon (IFN) regulatory factors (IRFs) and NF- κ B (nuclear factor κ B). These transcription factors (TFs) activate the expression of target genes, including IFNs, which in turn activate the transcription of a panel of IFN-regulated genes (IRGs) in a paracrine and autocrine manner (6). Many of these IRGs have known antiviral effects (7). Importantly, these host protective responses are dampened by the virus, for example, by the viral protease NS3-4A, which cleaves several key molecules in the IFN induction pathway, including the adaptor MAVS (5).

Many of these changes, whether metabolic reprogramming to facilitate viral replication or defense reactions, are accompanied or caused by transcriptional alterations of the infected cell (8, 9). Transcriptome-wide studies of HCV infection mostly focused so far on cell lines, in particular on the Huh-7.5 hepatoma cell line (10), which represents a robust *in vitro* infection model (e.g., see references 8 and 9). Transcriptional profiling of liver biopsy specimens from chronically infected patients was also performed (11). Previous studies in primary human hepatocytes (PHHs) were based on reverse transcription-quantitative PCR (RT-qPCR) or microarrays and restricted to specific gene subsets, in particular focusing on innate immunity (12–14). Responses to HCV infection in infected human hepatocytes at nearly single-cell resolution were described. However, these cells were derived from fetal liver cells rather than being directly explanted from an adult liver (15) and have an immature phenotype (16). Additionally, the initial phase of infection directly preceding infection was not captured.

In this report, we used RNA sequencing (RNA-seq) (17) to analyze the host transcriptional landscape directly after HCV infection of adult PHHs. Infection of human adult hepatocytes plated from liver resections of HCV-negative patients opens the unique possibility to study early time points directly after infection of natural HCV target cells, without contamination from other liver cell types (18), which could blur transcriptional signals. We compared the responses at 6 h postinfection (hpi) and 72 hpi in order to reflect the spatial transitioning of viral replication complexes from ribosomes (19) to the endoplasmic reticulum (ER) (20). In parallel, we also performed transcriptional profiling of highly permissive Huh-7.5 cells, which are widely used to propagate HCV *in vitro*, and directly compared HCV-induced gene dysregulation in the two systems for the first time.

FIG 1 Experimental protocol and RNA sequencing (RNA-seq) data validation. (A) Schematic of the experimental protocol. (B) Visualization of global transcriptional changes induced by HCV infection ($n=7$ donors) or treatment with UV-inactivated HCV (HCV^{UV}) ($n=4$ donors). For individual plots, average RPKM ($\log_{10}$) values for all detected transcripts from CM-treated cells are plotted on the x axes, with corresponding values from HCV-infected and HCV^{UV}-treated cells plotted on the y axes, respectively. Pearson's r and P values for each comparison are inset. (C) Three-dimensional principal-component analysis (PCA) from primary human hepatocyte (PHH) donor 4. Samples are distinguishable depending on their time point and infection status. (D) Control gene expression levels in PHHs and Huh-7.5 cells at 6 and 72 h postinfection (hpi). Heat maps of control gene RPKM values ($\log_{10}$) from individual PHH donors or different Huh-7.5 cell passages are shown. IRGs, interferon-regulated genes.

RESULTS

Experimental design and data visualization. Explanted adult PHHs from seven donors (donor 1 [D1] to D7) were plated and checked for viability prior to infection experiments. PHHs were incubated with conditioned medium (CM) or infected with replication-competent HCV (strain Jc1). Additionally, we inoculated PHHs with UV-inactivated HCV (HCV^{UV}) where sufficient viable patient material was available (D4 to -7). Cellular RNAs were isolated at two time points (6 hpi and 72 hpi) to enable monitoring of global hepatocyte transcriptional changes by RNA-seq and quantification of HCV RNA (Fig. 1A). Infection experiments in Huh-7.5 cells were also performed in parallel.

After mapping RNA-seq data to the hg38 genome scaffold, raw count data were normalized (reads per kilobase of transcript per million mapped reads [RPKM]) to allow comparison of global gene expression profiles within and between experiments. Mean RPKM values for all expressed genes were plotted for CM-treated PHHs versus HCV- or HCV^{UV}-infected PHHs, and correlation analyses were performed at both 6 hpi and 72 hpi (Fig. 1B). These analyses revealed that all comparisons were highly significant ($P<0.0001$), with Pearson's r correlation close to 1, indicating that the majority of

hepatocyte mRNAs are expressed at steady state and not significantly dysregulated upon HCV infection. Of note, Pearson's r was lowest at 72 hpi with replication-competent HCV (0.9566), indicating greater numbers of dysregulated hepatocyte genes under these conditions (Fig. 1B). Principal-component analyses (PCAs) were performed separately on samples from individual donors. While differences were observed between donors, separation of HCV-infected PHHs from CM/HCV^{UV}-treated cells and temporally distinct clusters at 6 and 72 h were generally observed (see an example plot from D4 in Fig. 1C). These analyses validate our approach of using time-matched infected and uninfected PHHs to avoid mixing of signals associated with HCV infection versus gradual hepatocyte dedifferentiation upon plating.

HCV infection activates antiviral defenses in PHHs but not Huh-7.5 cells. The transcript abundances of a panel of selected control genes were compared in PHHs and highly permissive Huh-7.5 cells (21), both with and without HCV infection. Comparable abundant expression of the hepatocyte marker *ALB* and transcripts encoding HCV entry and replication cofactors was detected in both PHHs and Huh-7.5 cells. Their expression remained stable across the experimental time course, suggesting no reduction in HCV permissiveness and no modulation of expression due to infection (Fig. 1D). As expected, the expression of lung-specific transcripts was either minimal or absent.

The baseline expression of a panel of IFN-regulated genes (IRGs) was detectable in PHHs, which was not further boosted upon infection with HCV^{UV} at either 6 or 72 hpi. While minimal IRG upregulation was observed upon infection with replication-competent HCV at 6 h, substantial induction was observed at 72 hpi. In contrast, the basal IRG expression level was demonstrably low or completely absent in Huh-7.5 cells compared to PHHs, and no induction was observed upon HCV infection at either sampling point.

Antiviral defenses in PHHs suppress HCV replication and completely abrogate virion release. To validate our RNA-seq data, we performed RT-qPCR on a selected set of test genes and compared the levels of gene induction in both systems. These data confirm a remarkable level of concordance between the two systems and confirm that our RNA-seq data accurately record both steady-state and virus-inducible gene expression (Fig. 2A). Due to the limitations of working with explanted hepatocytes, which include intact innate immune suppression of viral replication and limited sample material per donor, the percentage of infected PHHs could not be accurately determined. To investigate the effects of IRG induction on HCV RNA replication, viral RNA (vRNA) RT-qPCR was performed on cellular RNAs from both PHHs and Huh-7.5 cells (Fig. 2B). For replication-competent HCV, genome copy numbers did not increase between 6 and 72 hpi in PHHs, consistent with innate immune suppression of viral replication (Fig. 2B, top). In contrast, a 2-log increase in vRNA was apparent in Huh-7.5 cells over the same time course, consistent with a lack of IRG upregulation and unimpeded viral replication (Fig. 2B, bottom). As expected, minimal signal was detected in CM- or HCV^{UV}-treated cells. To determine the rates of infectious particle production, 50% tissue culture infective dose (TCID₅₀) titrations were performed in parallel on supernatants harvested from infected PHHs and Huh-7.5 cells (Fig. 2C). Titers at 6 hpi likely represent carryover of the initial inoculum despite extensive washing. While infectious virion secretion was completely absent in supernatants from PHHs at 72 hpi, HCV virions were detected at $\sim 1 \times 10^5$ TCID₅₀/ml in supernatants from Huh-7.5 cells at 72 hpi. To investigate this further, virion secretion was determined in PHHs that were pretreated with the JAK/STAT inhibitor ruxolitinib prior to infection. Ruxolitinib pretreatment rescued HCV virion release at 72 hpi, indicating that the ablation of virion production in PHHs is mediated by JAK/STAT-inducible immunity (Fig. 2C, top). Moreover, these results were confirmed via immunofluorescence staining for viral antigen. At 72 hpi, perinuclear NS5A localization in PHHs was visible only after JAK/STAT inhibition (Fig. 2D) but readily detected in Huh-7.5 cells without pharmacological immune suppression (Fig. 2E). Together, these data indicate that plated PHHs possess intact innate

FIG 2 Intact innate immunity suppresses HCV propagation in PHHs. (A) Comparative gene induction of four control genes measured by RT-qPCR or RNA-seq ($n=4$ donors). (Top) Fold gene induction determined by RT-qPCR and calculated by the $2^{-\Delta\Delta CT}$ method (43) compared to CM-treated PHHs. (Bottom) Fold gene induction based on RNA-seq data for the same samples under identical conditions. (B) Intracellular HCV RNA copies in HCV-infected PHHs ($n=4$ donors) (top) and Huh-7.5 cells ($n=3$) (bottom). Bars represent HCV RNA GE (genome equivalents) per $1 \mu\text{g}$ total RNA. *, P value of <0.05 ; ns, not significant. Data were log transformed, and a one-tailed, unpaired t test was applied to each comparison. (C) Secretion of infectious virions in supernatants of HCV-infected PHHs (top) and Huh-7.5 cells (bottom). Numbers of infectious particles in PHH supernatants at 72 hpi were under the LOQ of the assay at a 1:3 dilution. The dashed line indicates a separate experiment with PHHs from four additional donors, preincubated with $10 \mu\text{M}$ ruxolitinib (Rux), a JAK/STAT inhibitor, prior to and directly after HCV infection. (D and E) Immunofluorescence staining of viral NS5A protein in PHHs (D) or Huh-7.5 cells (E). PHHs were treated with CM or infected with HCV for 72 h with or without ruxolitinib pretreatment. NS5A staining is shown in red, with nuclear counterstaining (4',6-diamidino-2-phenylindole [DAPI]) in blue.

immunity, which suppresses initial HCV replication and completely blocks virion release. This inducible immunity is absent in Huh-7.5 cells.

HCV infection of PHHs dysregulates gene programs associated with diverse biological functions. Statistical analyses were performed to quantify differentially expressed genes (DEGs) induced upon HCV infection of PHHs (false discovery rate [FDR] P value of <0.05). For replication-competent HCV infections, a temporally structured increase in DEGs was observed (Fig. 3A, left, and Fig. 3B), which partially overlapped (Fig. 3C). At 6 hpi, 81 infection-induced DEGs were apparent; this number was markedly amplified at 72 hpi (DEGs, $n=2,985$) and indicates that HCV infection ultimately induces a substantial shift in the PHH transcriptional landscape (Fig. 3A, left, and Fig. 3B). For HCV^{UV} infections, a contrasting temporally structured reduction in

FIG 3 HCV infection induces temporally structured and functionally diverse gene programs in PHHs. (A) Comparison of the numbers of HCV- and HCV^{UV}-induced DEGs (left) and enriched GO categories (right) at 6 and 72 hpi. (B) Genomic location of HCV-induced transcripts. Manhattan plots compare transcript abundances in uninfected versus HCV-infected PHHs at 6 hpi (top) and 72 hpi (bottom). Circles represent individual gene comparisons, with sizes proportional to average RPKM fold

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DEG signatures was observed: an initial transcriptional response to HCV^{UV} was detected at 6 hpi ($n=239$), which declined to virtually no detectable dysregulation at 72 hpi ($n=12$) (Fig. 3A, left).

To determine the biological processes associated with HCV-induced DEGs, gene ontology (GO) enrichment analyses were performed. At 6 hpi, HCV- and HCV^{UV}-enriched GO categories were associated with shared and distinct biological processes (Fig. 3A, right, and Fig. 3D, top two panels). Of note, only PHHs infected with replication-competent HCV demonstrated enrichment of GO categories associated with IFN signaling or pathogen defense: dysregulation of these gene classes was absent in HCV^{UV}-infected PHHs. These data represent the early yet restricted signatures of the hepatocyte antiviral response and confirm that this response is induced only by replication-competent vRNAs.

Proportional to the quantity of HCV-induced DEGs, a large number of highly significantly enriched GO categories were identified at 72 hpi, which were associated with diverse biological processes (Fig. 3A, right, and Fig. 3D, bottom). The overlap with DEGs representing described IRGs ($n=390$) (7, 22) or IRG interactors ($n=2,582$) (22) (Fig. 3C, bottom) resulted in the enrichment of multiple GO categories associated with antiviral responses and innate immunity (Fig. 3D, bottom). However, many DEGs were not classical IRGs/IRG interactors and represent biological processes not automatically associated with the antiviral response (e.g., ribosome biogenesis, translation initiation, and protein targeting to the ER) (Fig. 3D, bottom). Examples of opposing patterns of gene dysregulation can be seen in Fig. 3E for two representative GO categories: while genes associated with defense response to virus are generally strongly upregulated at 72 hpi, genes associated with protein targeting to the ER are almost exclusively downregulated. Together, these early snapshots of virus-induced cellular changes confirm temporally regulated transcriptional responses to HCV infection in adult PHHs, with the magnitude of gene induction increasing exponentially from 6 to 72 hpi.

Divergent transcriptional responses to HCV infection and ectopic *IRF1* expression in Huh-7.5 cells. Huh-7.5 cells represent the most frequently used cell line for HCV propagation in research. Consequently, we also cataloged global HCV-induced transcriptional responses in these cells for comparative purposes. Similar to PHHs, statistical analyses identified a time-dependent increase in significant HCV-induced DEGs and enriched GO categories (Fig. 4A).

Intrinsic *IRF1* expression has been reported to maintain the baseline transcription of a program of antiviral genes, independently of the IFN system (23, 24). To quantify the *IRF1* regulon in human cells of hepatic origin, we transcriptionally reprogrammed Huh-7.5 cells by ectopically expressing *IRF1*. DEGs and GO enriched categories induced by HCV at 72 hpi and ectopic *IRF1* expression were numerically similar (Fig. 4A and B). However, the underlying dysregulated genes exhibited an $\sim 10\%$ overlap (Fig. 4C, top), and the significantly enriched biological processes associated with the dysregulated genes were highly divergent (Fig. 4D). HCV-induced genes in Huh-7.5 cells were associated with diverse biological processes, including the circadian rhythm or amino acid transport (Fig. 4D, top), and showed minimal overlap with HCV-induced genes in PHHs (Fig. 4C, bottom). In contrast, *IRF1*-regulated genes were largely but not exclusively associated with GO categories related to innate immunity or pathogen defense (Fig. 4D, bottom). Examples of *IRF1*-mediated gene dysregulation are shown for two representative GO categories associated with innate immunity (Fig. 4E). In summary, these data expand the *IRF1* regulon identified by gene microarrays previously (7) ($n=130$) by an additional 329 genes ($n=459$) and confirm that Huh-7.5 cells retain the capacity to

FIG 3 Legend (Continued)

changes ($\log_2$). (C) Number of HCV-induced genes in PHHs at 6 and 72 hpi. The top Venn diagram shows the overlap between DEGs at 6 and 72 hpi, while the bottom diagram shows the gene overlap at 72 hpi with characterized IRGs (*) (7) and IRG interactors (**) (22). Only DEGs with FDR P values of <0.05 and RPKM values of >1 were included. (D) GO enrichment analysis of HCV-induced DEGs in PHHs. Each circle represents a GO category and is plotted depending on its P value and size. The size illustrates the frequency of the GO term in the underlying GO annotation (46). Representative GO categories are highlighted in graphs, and their full annotation is displayed below each plot. (E) HCV-induced transcripts at 72 hpi. Mean ($n=7$ donors) fold changes (FC) ($\log_2$) of HCV-induced DEGs associated with the defense response to virus (top) or protein targeting to the ER (bottom) are shown.

FIG 4 Distinct *IRF1*- and HCV-mediated transcriptional programs in Huh-7.5 cells. (A) Comparison of numbers DEGs (left) and enriched GO categories (right) upon HCV infection or ectopic *IRF1* expression in Huh-7.5 cells. (B) Genomic location of HCV-induced (top) or *IRF1*-induced (bottom) transcripts in Huh-7.5 cells. Manhattan plots compare the transcript abundances in uninfected Huh-7.5 cells to those in HCV-infected or *IRF1*-expressing cells. Circles represent individual gene comparisons, with sizes proportional to average RPKM fold changes ($\log_2$). (C) HCV- and *IRF1*-induced DEGs show limited overlap. The top Venn diagram shows the overlap between HCV- and *IRF1*-induced DEGs, while the bottom diagram shows the overlap of HCV-induced DEGs in Huh-7.5 versus PHHs. Only DEGs with FDR P values of < 0.05 and RPKM values of > 1 were included. (D) GO enrichment analyses of HCV- and *IRF1*-induced DEGs in Huh-7.5 cells. Each circle represents a GO category and is plotted depending on its P value and size. The size illustrates the frequency of the GO term in the underlying GO annotation (46). Representative GO categories are highlighted in graphs, and their full annotation is displayed below each plot. (E) *IRF1*-induced transcripts in Huh-7.5 cells. Mean fold changes ($\log_2$) of *IRF1*-induced DEGs associated with the innate immune response (top) or regulation of intracellular signal transduction (bottom) are shown.

FIG 5 Ectopic *IRF1* expression induces an antiviral gene signature in Huh-7.5 cells similar to baseline expression in PHHs. (A) Baseline expression of IRGs and *IRF1*. (Top) Basal IRG expression in Huh-7.5 cells with and without *IRF1* expression, compared to uninfected PHHs. (Bottom) Comparison of baseline expression of *IRF1* and pattern recognition receptors (PRRs) that recognize double-stranded RNA (dsRNA) in Huh-7.5 cells and PHHs. (B) Area under the curve (AUC) analysis. Shown are differences in *IRF1*-regulated gene expression levels for two distinct GO categories from Huh-7.5 EMPTY and Huh-7.5 *IRF1* cells, compared to expression in PHHs. Higher AUC values indicate more divergent expression profiles. (C) Correlation plots of *IRF1*-regulated genes from the same two GO categories. RPKM values are plotted for individual genes, and simultaneous comparison of their expression in uninfected PHHs to those in both Huh-7.5 EMPTY and Huh-7.5 *IRF1* cells is visualized. Pearson's r and P values for each comparison are inset. (D) HCV-inducible *IRF1*-regulated genes in PHHs at 72 hpi. +, mean log₂ fold change in expression. (E) Restriction of HCV replication and translation by ectopic *IRF1* expression. Huh-7.5 EMPTY and Huh-7.5 *IRF1* cells were electroporated with a subgenomic replicon of JFH-1. h.p.e., hours postelectroporation. ****, $P<0.0001$. Data were log transformed, and multiple unpaired t tests were performed comparing Huh-7.5 EMPTY versus Huh-7.5 *IRF1* cells and Huh-7.5 EMPTY cells with DMSO versus Huh-7.5 EMPTY cells with 2'CMA, respectively.

mount antiviral defenses. Our highly sensitive RNA-seq profiling identified 271 additional low-abundance transcripts as significantly dysregulated (FDR P values of <0.05 ; final RPKM value of <1), although these were omitted from subsequent analyses to increase stringency.

Baseline *IRF1* expression coordinates an intrinsic antiviral program in PHHs.

Using our previously selected panel of control genes (Fig. 1D), we visualized the baseline expression of IRGs in uninfected Huh-7.5 cells transduced with an empty lentivirus (Huh-7.5 EMPTY), Huh-7.5 *IRF1* cells, and uninfected PHHs. We observed highly similar baseline IRG expression levels in Huh-7.5 *IRF1* cells and uninfected PHHs, distinct from that in Huh-7.5 EMPTY cells (Fig. 5A, top). We also visualized the baseline expression of *IRF1* in Huh-7.5 cells and PHHs and compared the expression levels to those of pattern recognition receptors (PRRs) that recognize cytosolic double-stranded RNA (dsRNA) (Fig. 5A, bottom). These data confirm minimal *IRF1* expression in Huh-7.5 cells, 4-fold lower than PHH baseline expression levels. These observations further reveal much higher baseline expression levels of the cytosolic dsRNA sensors *TLR3*, *DDX58* (RIG-I), and *IFIH1* (MDA5) in PHHs than in Huh-7.5 cells.

Next, we compared the mean expression levels of *IRF1*-regulated genes from both Huh-7.5 EMPTY and Huh-7.5 *IRF1* cells to the expression levels in PHHs. Applying robust statistical methods to compare gene subsets from two distinct enriched GO categories within the *IRF1* regulon, we performed area under the curve (AUC) and correlation analyses. AUC analyses confirmed that the expression of *IRF1*-regulated genes in Huh-7.5 *IRF1* cells was more closely related to baseline expression in PHHs than in Huh-7.5 EMPTY cells (Fig. 5B). Of note, this pattern was more pronounced for genes associated with immune system processes (GO:0002376) than for genes associated with the regulation of metabolic processes (GO:0019222). Correlation analyses compared the mean expression levels of individual *IRF1*-regulated genes in PHHs to those in both Huh-7.5 EMPTY and Huh-7.5 *IRF1* cells (Fig. 5C). While all comparisons were significantly correlated ($P < 0.0001$), higher Pearson's r values were observed for Huh-7.5 *IRF1* cells ($r = 0.52$ and $r = 0.53$) than for Huh-7.5 EMPTY cells ($r = 0.36$ and $r = 0.42$), indicating that ectopic expression of *IRF1* pushes the Huh-7.5 cell transcriptome to a more PHH-like state. Correspondingly, this pattern was again more pronounced for immune system process genes (GO:0002376) than for genes associated with the regulation of metabolic processes (GO:0019222) (Fig. 5C). *IRF1* also represents an IRG, and its expression is further boosted by HCV infection (Fig. 3E, top). Consequently, we next determined what proportion of the *IRF1* regulon is further dysregulated upon HCV infection of PHHs (Fig. 5D). These analyses identify 178 *IRF1*-regulated genes whose expression is significantly dysregulated by HCV infection of PHHs, indicating that *IRF1* also contributes to inducible immunity.

To determine the effect of ectopic *IRF1* expression on HCV RNA replication, we transfected either Huh-7.5 EMPTY or Huh-7.5 *IRF1* cells with a subgenomic replicon (SGR) containing the nonstructural proteins NS3 to NS5B from strain JFH-1 coupled to a firefly luciferase (F-luc) reporter and monitored luciferase accumulation over time (Fig. 5E). These experiments confirm that the transcriptional program mediated by *IRF1* has the ability to reduce HCV RNA replication significantly. We also performed transfections in the presence of the HCV replication inhibitor 2' C-methyl adenosine (2'CMA) or a dimethyl sulfoxide (DMSO) vehicle control. The significant *IRF1*-mediated reduction in relative luciferase units (RLU) was also apparent at 4 h posttransfection in Huh-7.5 *IRF1* cells, which was not the case in 2'CMA-treated cells, indicating that the *IRF1* gene program also negatively impacts viral genome translation, possibly due to direct targeting or competition with host IRG mRNAs for available ribosomes (Fig. 5E). In summary, these analyses provide supportive evidence that *IRF1* coordinates the baseline expression of antiviral effector genes in PHHs in the absence of infection and also contributes to inducible immunity. The antiviral effect mediated by the *IRF1* gene program was able to reduce HCV replication/translation by 1 to 2 logs. Thus, in addition to the observed triggering of antiviral defenses by HCV, it is likely that this suite of intrinsically expressed genes in PHHs contributes to the defense against incoming virus.

Comparison of transcriptional regulators and targeted canonical pathways. To determine upstream transcriptional regulators that orchestrate changes in gene expression induced by HCV infection of PHHs or Huh-7.5 cells or ectopic *IRF1* expression in Huh-7.5 cells, we performed ingenuity upstream regulator analysis (Qiagen). Focusing on nuclear transcription factors (TFs), we observed significant activation (Fig. 6A, blue) or inhibition (orange) ($P < 0.05$) of diverse transcriptional regulators, with distinct and overlapping TF profiles observed for each data set. Limited overlap ($n = 5$) was observed between the TFs of coordinating transcriptional responses to HCV infection in PHHs and Huh-7.5 cells. Of note, low oxygen tension has been demonstrated to enhance HCV RNA replication (25), and activation of hypoxia-inducible factor 1 alpha (HIF1A) is observed in both systems, which is a known regulator of transcriptional responses to hypoxia (26).

As expected, considerable overlap was observed between the transcriptional regulators coordinating the observed patterns of gene dysregulation detected in HCV-infected PHHs and *IRF1*-reprogrammed Huh-7.5 cells, as *IRF1* is further upregulated upon HCV infection of PHHs. Multiple shared TFs known to activate antiviral programs

FIG 6 Upstream transcriptional regulators and canonical pathways dysregulated by HCV infection or ectopic *IRF1* expression. (A) Upstream transcriptional regulators controlling DEGs. Ingenuity pathway analysis (IPA) was used to calculate upstream regulators based on significant DEGs identified under the three presented conditions. Z-scores indicate activation or inhibition of individual regulators. Displayed are only the transcriptional regulators with a *P* value of <0.05 and a Z-score higher than 2 or lower than -2. (B) Canonical pathway analyses of HCV- and *IRF1*-induced DEGs in PHHs and Huh-7.5 cells, respectively. Pathways are plotted with the corresponding *P* values (bars) and ratios between dysregulated molecules in our data sets and all molecules belonging to that pathway (linked black circles). The dotted line represents the significance threshold (*P*=0.05). The bar color represents the expression Z-score. LPS, lipopolysaccharide; IL-1, interleukin-1. (C) Visualization of the top HCV-dysregulated pathway in PHHs at 72 hpi (eIF2 signaling) and its underlying DEGs. (Left) Modified cartoon of eIF2 signaling as determined by IPA. (Right) Heat map displaying fold changes of significant DEGs involved in this pathway. GAP, GTPase-activating protein; uORF, upstream open reading frame; ITAFs, internal ribosome entry site (IRES)-transacting factors.

were activated. Furthermore, these analyses demonstrate that ectopic *IRF1* expression additionally activates an array of transcriptional regulators, resulting in a broad induction of antiviral effectors, and highlight that *IRF1* coordinates a complex web of multiple TFs, which collectively contribute to the *IRF1* regulon.

To further explore these data, we used ingenuity pathway analysis (IPA) (Qiagen) to investigate which canonical cellular pathways are affected by HCV- or *IRF1*-mediated gene dysregulation. Shared and distinct canonical pathways were targeted in all three systems (Fig. 6B [only the top 10 hits are presented]). Notably, for HCV infection of PHHs, a number of targeted pathways were associated with innate immune responses, including “interferon signaling,” “antigen presentation pathway,” and “activation of cytosolic IRF by PRRs.” Targeted transcriptional dysregulation of pathway components associated with IFN-mediated innate immunity was absent in HCV-infected Huh-7.5 cells. As Huh-7.5 cells do not produce IFN and possess impaired antiviral effector responses, we reasoned that these HCV-targeted pathways represent proviral transcriptional manipulation to facilitate HCV propagation. Cell-intrinsic pathways targeted by *IRF1* were generally involved in innate immunity and exhibited some crossover with HCV-infected PHHs, including “interferon signaling,” “antigen presentation pathway,” and “complement system.”

In HCV-infected PHHs, in addition to pathways associated with classical IFN-mediated innate immunity, we also observed targeting of unrelated pathways, including “eIF2 signaling,” “mitochondrial dysfunction,” and “mTOR signaling.” The top targeted pathway in HCV-infected PHHs was “eIF2 signaling,” and detailed inspection of this pathway identified significantly dysregulated molecules at multiple pathway stages (Fig. 6B, left). The corresponding changes in gene expression for targeted molecules within the “eIF2 signaling” pathway highlight the significant downregulation of genes that comprise the structural components of ribosomes and the translation preinitiation complex. In contrast, significant upregulation of the protein kinase R gene (*PKR*) and *MYC* is observed (Fig. 6B, right). Together, these analyses provide a broad overview of the transcriptional regulators that orchestrate HCV- or *IRF1*-mediated gene dysregulation and the downstream cell-intrinsic pathways that they target.

DISCUSSION

In this study, we sought to quantify and dissect initial global transcriptional responses to HCV infection of authentic target cells: adult PHHs. Acquiring these data *in vivo* is particularly challenging. Indeed, HCV has a highly restricted cellular tropism and efficiently infects only human hepatocytes. The liver is a solid organ that is composed of multiple cell types (27), and while hepatocytes represent the major cell type, these cells are not readily accessible for sampling. Acute HCV infection is also often asymptomatic, so initial infections often go unnoticed. Here, we sought to overcome these hurdles by performing *ex vivo* infections of PHHs isolated from adult donors who were not previously infected with HCV or treated with IFN. Our quantification of initial acute-phase responses to infection in adult PHHs provides a snapshot of early perturbations in the hepatocyte transcriptional landscape induced by HCV infection.

In agreement with the reported spatiotemporal shifting of early HCV replication complexes within the cytosol, patterns of HCV-induced gene dysregulation were time structured in adult PHHs. Limited gene induction was observed at 6 hpi, where initial viral replication complexes are associated with ribosomes (19). However, weak induction of a restricted panel of IRGs was detected, which were not upregulated in HCV^{UV}-treated cells, and represents the first signatures of the PHH antiviral response to replication-competent HCV. This limited induction of antiviral effectors shortly after infection could represent HCV NS3/4A-mediated targeting of MAVS, which dampens host antiviral responses (28), and contrasts with IFN or poly(I:C) treatment of PHHs, where early DEG induction at 6 h is associated with broad IFN-mediated responses (29). Alternatively, intrinsic *IRF1* expression in PHHs may limit the replication capacity of incoming virus (see below for further discussion), reducing the accumulation of dsRNA and therefore delaying broad innate immune induction. In contrast to 6-hpi DEGs, the spatial transitioning of viral replication complexes to remodeled ER membranes at 72 hpi (20) coincided with an exponential amplification of HCV infection-mediated transcriptional dysregulation. We detected significant dysregulation of ~3,000 genes, associated with a diverse array of biological processes.

At 72 hpi in PHHs, we observed expansive induction of IFN-triggered antiviral effector genes, promoting the suppression of viral replication and the abrogation of particle release. However, 80% of HCV-infected individuals fail to mount effective responses, facilitating the progression to chronicity, and viruses have evolved a variety of innate immune evasion strategies to promote their propagation, which includes host translational shutoff (30). Indeed, while HCV infection results in effective IRG induction, virus-induced phosphorylation of PKR inhibits the α subunit of eukaryotic initiation factor 2 (eIF2 α) and therefore blocks host IRG protein translation (31). PKR activation is therefore advantageous to HCV and prevents clearance because while the translation of capped host mRNAs is dependent on eIFs, HCV polyprotein translation occurs independently of eIFs using an internal ribosome entry site (IRES) located in the 5' untranslated region (UTR) of the viral genome (32). Along with the previously described HCV inhibition of eIF2 α (31), our transcriptional profiling identifies broad downregulation of multiple components of the host translational machinery. Mechanistically, this process is likely mediated by the TF MYC, which is known to directly regulate ribosome biogenesis and translation, controlling the expression of the RPS and RPL proteins of the small and large ribosomal subunits, in addition to the gene products necessary for rRNA processing, nuclear export of ribosomal subunits, and mRNA translation initiation (33). Interestingly, in PHHs, significant upregulation of *MYC* mRNA results in the downregulation of the gene products that it controls (Fig. 6C, right). These data may represent a previously unappreciated mechanism whereby the downregulation of constituent components of the translational machinery arrests the translation of antiviral effector genes. In contrast, MYC is predicted to be inhibited in upstream regulator analysis (Fig. 5A). Detailed investigation of the genes involved in the predicted MYC inhibition and the interactions included in the IPA knowledgebase highlights a number of genes involved in IFN signaling based on microarray analysis in plasmacytoid dendritic cells (pDCs) (34). These genes are highly upregulated upon *MYC* knockdown in pDCs, and the same genes are induced in the antiviral immune response in PHHs upon HCV infection. Differences between cellular responses in pDCs and PHHs or upon knockdown of MYC compared to HCV infection may result in the upregulation of *MYC* in our data set. Together, these data simultaneously capture infection-induced transcriptional signatures associated with proviral translational shutoff and antiviral IFN signaling.

Furthermore, we observed transcriptional dysregulation of additional gene programs that have been reported to modulate susceptibility to HCV. We observed targeting of the mTOR signaling pathway, which has been reported as proviral, with the mTOR inhibitor rapamycin targeting HCV replication *in vitro* and reducing viral RNA levels in patients posttransplantation (35). HCV infection has also been shown to activate mTOR, and pharmacological inhibition of mTOR was shown to suppress HCV virion assembly and release *in vitro* (36). Of note, significant targeting of multiple pathways associated with nuclear receptor (NR) signaling was detected in both PHHs and Huh-7.5 cells, further confirming that Huh-7.5 cells retain some HCV-inducible programs that are of biological relevance. NR-mediated signaling regulates transcriptional programs that control host metabolic processes and lipid metabolism and are reported to modulate susceptibility to HCV infection in both humans (37) and mice (38).

In addition to HCV activation of pro- and antiviral cascades, we also investigated the contribution of *IRF1*-mediated intrinsic immunity in PHHs to the control of HCV replication. *IRF1* is a TF that participates in IFN induction but also directly induces a subset of IRGs. Additionally, *IRF1* represents a potent panviral restriction factor and a key component of the cellular antiviral response (7). Using microarray analysis, Schoggins et al. identified a panel of 130 partially overlapping, *IRF1*-regulated genes (>3-fold) via lentiviral overexpression in Huh-7 and *STAT1*^{-/-} fibroblasts (7). More recently, it has been demonstrated that constitutive expression of *IRF1* in immortalized PH5CH5 cells of hepatic origin (24) and BEAS-2B bronchial epithelial cells (23) coordinates intrinsic antiviral protection independently from the IFN system. Ectopic expression of *IRF1* in Huh-7.5 cells enabled us to define a greatly expanded *IRF1* regulon, and comparative statistical analysis of Huh-7.5 EMPTY, Huh-7.5 *IRF1*, and uninfected PHH

transcriptomes provides supportive evidence that baseline immunity in PHHs in the absence of infection is orchestrated by *IRF1*. Previous studies have shown *IRF1* to be a potent restrictor of HCV replication (7). We confirm this observation and also determine that *IRF1* can significantly inhibit HCV subgenome translation in Huh-7.5 cells. While intrinsic *IRF1* expression in PHHs maintains a suite of genes that can actively suppress HCV translation and replication, which may contribute to the low levels of gene induction at 6 hpi, this translational suppression could be partially overcome by the host translational shutoff that we observe at 72 hpi.

In summary, the virus-host interactions that determine the susceptibility of human hepatocytes to initial HCV infection and their capacity to support persistent infection are incompletely defined. The high levels of genetic diversity seen between HCV genotypes point to a long period of association for virus and host coevolution (39), and HCV has evolved multiple strategies to hijack the host cell machinery required to facilitate its propagation while at the same time evading host defenses. We observe early concurrent transcriptional dysregulation of gene programs that facilitate viral persistence, balanced against those that promote viral inhibition. This cellular pro- and antiviral antagonism may keep HCV replication levels below a clearance threshold in the initial phase of infection and ultimately facilitate progression to chronicity.

MATERIALS AND METHODS

Source of PHHs. PHHs were isolated from surgical liver resections as previously described (18). PHHs were obtained with informed consent approved by the ethics commission of Hannover Medical School (Ethik-Kommission der MHH, number 252-2008). Additionally, PHHs were commercially obtained (Lonza, Basel, Switzerland). Cryopreserved PHHs were thawed as recommended by the manufacturer.

Virus production and UV inactivation. Huh-7.5 cells were electroporated with HCV genomic RNA transcripts (Jc1 strain) (40, 41), and supernatants were harvested at 48, 72, and 96 h postelectroporation, filtered (0.45- μ m pores), pooled, aliquoted, and frozen at -80°C . A portion of this virus stock was UV inactivated in a 6-well dish with 1 ml per well at 5 J/cm².

Generation of Huh-7.5 *IRF1* cells. The *IRF1* coding sequence (gBlock; IDT) was cloned into lentiviral vector pWPI-bla (Addgene) and confirmed by Sanger sequencing (GATC). Cotransfection of plasmids encoding vesicular stomatitis virus G protein (VSV-G) and HIV-1 Gag/Pol and plasmid pWPI-bla-*IRF1* into HEK293T cells was performed using Lipofectamine 2000 (Invitrogen). Supernatants containing lentiviral pseudoparticles for transgene delivery were harvested at 24 h and 48 h, pooled, filtered (0.45- μ m pores), and used to transduce 2×10^5 Huh-7.5 cells. At 72 h posttransduction, blasticidin (10 μ g/ml) was added to media. For control purposes, Huh-7.5 cells were also transduced with pseudoparticles containing an empty pWPI-bla vector (no transgene expressed). After blasticidin addition, surviving cells were expanded via passaging for 14 days prior to freezing at -150°C with 10% DMSO.

HCV infection and RNA isolation. PHH infections were performed at a multiplicity of infection (MOI) of 1, calculated using Huh-7.5 cells, and the same volume was used for treatment with UV-inactivated virus. At 4 h postinoculation, cells were washed with phosphate-buffered saline (PBS), and 1 ml of fresh hepatocyte culture medium (HCM) was added per well (Lonza). At 6 or 72 hpi, supernatants were collected, and cells were lysed in 1 ml TRIzol reagent (Invitrogen) or RA1 buffer supplemented with β -mercaptoethanol (Macherey-Nagel). All samples were stored at -80°C until processing. Total RNA was extracted according to the manufacturer's instructions. For infection of Huh-7.5 empty vector-transduced cells, identical conditions were used except that Dulbecco's modified Eagle's medium (DMEM) was used instead of HCM and RNA extractions were performed using only the NucleoSpin RNA kit (Macherey-Nagel). Where ruxolitinib treatment is indicated, PHHs were pretreated with 10 μ M ruxolitinib on the day before HCV infection. For the rescue of viral particle production (Fig. 2C), 10 μ M ruxolitinib was also added to HCM directly after infections.

Subgenomic replicon assays. Huh-7.5 cells expressing *IRF1* or transduced with an empty lentivirus were electroporated with equal amounts (2.5 μ g) of a firefly luciferase (F-luc)-expressing subgenomic replicon RNA (NS3-NS5B, strain JFH-1) and seeded onto 12-well dishes. Additionally, empty control cells were treated with 1 μ M 2'-CMA or DMSO. Cells were lysed at 4, 24, 48, 72, and 96 h postelectroporation in 350 μ l passive lysis buffer (Promega) per well and frozen at -20°C until measurement of F-luc expression with a tube luminometer.

TCID₅₀ and RT-qPCR. Viral titers in cellular supernatants and viral stocks used for infection experiments were quantified on Huh-7.5 cells using a limiting dilution assay as previously described (42). The limit of quantification (LOQ) was determined by the lowest, still evaluable result in the assay setup. One-step RT-qPCR was performed to determine intracellular HCV RNA copy numbers using the LightCycler 480 RNA master hydrolysis probes kit (Roche) and an HCV-specific primer-probe set targeting the 5' UTR. RT-qPCR was performed using a LightCycler 480 instrument (Roche). Ten RNA copies was the lowest number used to calculate the standard curve.

To determine the relative gene expression of selected cellular genes, 250 ng of total cellular RNA was reverse transcribed using the TaKaRa reverse transcription (RT) kit. TaKaRa's SYBR Premix ExTaq II was used according to the manufacturer's instructions, with gene-specific primers. RT-qPCR was

performed using a LightCycler 480 instrument (Roche). Changes in relative gene expression were calculated according to the $2^{-\Delta\Delta CT}$ method (43).

RNA-seq. Cellular RNAs were used to generate sequencing libraries using a ScriptSeqv2 kit (Illumina) and run on the Illumina HiSeq 2500 platform, and subsequent data analyses were performed using CLC Genomics Workbench (Qiagen, Aarhus, Denmark) (44). Mapping against the human reference genome (hg38) was performed for individual samples, and relative transcript expression was calculated from raw count data via normalization to gene length (RPKM) (45). Identification of differentially expressed genes (DEGs) was conducted by comparing raw count data, with calculation of the false discovery rate (FDR) *P* values for multiple comparisons. Significant DEGs with low expression levels (FDR *P* values of <0.05 with a final RPKM value of <1) were omitted from subsequent GO, TF, and pathway analyses.

GO enrichment analyses. GO analyses were performed using the GO Resource (<http://geneontology.org/>). ENSEMBL identifiers for DEGs were used as the input, and identification of significantly enriched GO categories was performed using the Panther classification system (<http://pantherdb.org/>). *P* values for specific GO categories were generated after Bonferroni correction for multiple testing. Redundant GO terms were removed with REVIGO (46) (<http://revigo.irb.hr>), with the following settings: allowed similarity “tiny,” GO term sizes database “*Homo sapiens*,” and “SimRel” semantic similarity measure. The remaining terms were visualized in semantic similarity-based scatterplots using GraphPad Prism v9.0.

Canonical pathway analysis and transcriptional regulators. Pathway analyses were performed using ingenuity pathway analysis (Qiagen, Aarhus, Denmark) (47). Input data included ENSEMBL identifiers, FDR *P* values, and fold change values for DEGs determined using CLC Genomics Workbench. Default settings were used for all categories except for species, which was set to human. Z-scores were calculated based on the expression fold change. IPA compares input data sets with the ingenuity knowledgebase, which represents a collection of published interactions of molecules. Upstream regulators are determined bioinformatically (http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf). Analysis outputs were exported as Excel sheets or PDF files, and data were replotted with GraphPad and Adobe Illustrator. Disease-, cancer-, and non-hepatocyte-associated pathways were omitted from the presented diagrams.

Data availability. RNA-seq data generated in this study and subsequent downstream analyses, including the identification of DEGs, enriched GO categories, upstream TFs, and targeted pathways, were submitted to the NCBI GEO database under accession number [GSE166428](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166428).

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