

An RNAi screen identifies cellular requirements for the later stages of Hepatitis B virus replication

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ABSTRACT

Hepatitis B virus (HBV) is a devastating human pathogen with upwards of 250 million people chronically infected. HBV is a leading cause of end-stage liver disease (cirrhosis) and hepatocellular carcinoma. Due to the inability of current treatments to eliminate the HBV reservoir and the frequent occurrence of resistant strains, additional therapies are required. Toward this goal we sought to identify novel host factors required for HBV replication using a whole genome RNAi screen in cells constitutively infected with HBV. This screen identified 69 novel high-confidence candidates which modulate HBV replication. Of these, four were found to be required for viral transcription, BRCA1, SIRT2, YWHAH, and ZCCHC14. Moreover, YWHAH and ZCCHC14 were found to be required for the productive infection in clinical HBV isolates of both transformed cells and primary human hepatocytes. Our siRNA screen discovered several HBV host factors that contribute to productive HBV replication and thus represent potential targets for anti-HBV therapies.

KEYWORDS: Hepatitis B virus, HBV, HBV host factors, siRNA screen, SIRT2, YWHAH, ZCCHC14.

INTRODUCTION

HBV is an enveloped partially double-stranded DNA hepadnavirus that infects hepatocytes subsequent to viral exposure of the host's mucous membranes or circulatory system. In adults, HBV typically causes transient acute hepatitis; however, 5% of these infections become chronic, and these can progress to cirrhosis, hepatocellular carcinoma (HCC), and death. Infected neonates acquire HBV via vertical transmission from their mothers and this results in high rates of chronic infection (>90%) and a 35-40% increased risk of HCC and/or cirrhosis [1]. Though an effective vaccine exists, it is estimated that nearly 2 billion individuals have been infected and, of those, 250 million have chronic infections [2]. While highly effective antiviral treatments for chronic HBV-infected patients are available, they fail to eliminate viral reservoirs [3] and, therefore, patients, require lifelong therapy with the risk of developing drug resistance and/or HCC (the latter occurring also in the absence of cirrhosis). Consequently, an improved understanding of the HBV life cycle and the

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identification of new therapeutic targets for chronic HBV infection are required.

The study of HBV biology has been challenged by the lack of a robust HBV cell culture system as well as the absence of previous genetic screens. Despite the recent discovery of HBV host receptor, the sodium-taurocholate co-transporting polypeptide (NTCP) [4], the early events of HBV life cycle remain poorly characterized. Post-entry, the viral nucleocapsid docks on the cytosolic face of the nuclear pore complex, where it undergoes uncoating, permitting the release of the viral DNA genome into the nucleus [5]. The viral genome, referred to as the relaxed circular rcDNA, is comprised of a unit-length negative strand and a partially complete positive strand [6]. Within the nucleus, the partial positive DNA strand is completed and the genome circularized by host enzymes to form the covalently closed circular DNA (cccDNA) [7]. The cccDNA in turn serves as the template for the synthesis of viral mRNAs.

Expression of the viral genes is controlled by a combination of two enhancers, four promoters, and a regulatory element [8]. Through the engagement of alternative start sites, the mRNAs are translated into five HBV proteins – a protein of unknown but possibly immunoregulatory function, HBeAg; the L, M, and S forms of the envelope protein, HBsAg; the transcriptional regulator, HBx; the structural protein, core; and the reverse transcriptase and DNA polymerase, Pol [5]. One transcript, termed the pregenomic mRNA (pgRNA) serves as the template used in a reverse-transcription event that produces progeny genomes [7]. In the cytoplasm, the core protein forms the nucleocapsid and associates with pgRNA. Within these capsids, Pol covalently links to pgRNA and reverse transcribes it to give rise to the rcDNA. Capsids can then reenter the nucleus, amplifying or maintaining the presence of cccDNA, or undergo envelopment and transit through the Golgi with their subsequent release into the hepatic sinusoids of the infected individual [7].

With its compact genome, HBV is reliant upon host factors for many critical aspects of its replication. While two HBV-encoded proteins - HBx and the L-protein - act, in part, to transactivate HBV gene expression [9, 10], neither appears to

bind directly to viral DNA and HBV depends on the activity of cellular factors for the transcription and translation of its mRNAs. As HBV replicates solely in hepatocytes, it makes use of liver-enriched or -restricted transcription factors. In particular, HBV transcription is influenced by several of transcription factors that control the metabolic state of hepatocytes, including hepatocyte nuclear factor 1 (HNF1), HNF4 α , peroxisome proliferator-activated receptor (PPAR) alpha, PPAR γ coactivator 1-alpha (PGC1 α), farnesoid X receptor alpha, liver receptor homologue 1, forkhead box protein O1, cAMP response element-binding protein, CCAAT-enhancer-binding protein α , and glucocorticoid receptor (GR) [11-15]. Besides restricting HBV replication to hepatocytes, the virus' reliance on these transcription factors also integrates HBV transcription with the host cell's metabolic signaling pathways. For instance, treatment of hepatocytes with forskolin, which raises intracellular cAMP levels and activates the gluconeogenesis pathway, stimulates HBV transcription by promoting interaction between CREB regulated transcription coactivator 2 (CRT2) and PGC1 α [14]. The HBV genome lacks introns. Common to all HBV transcripts is a region known as the posttranscriptional regulatory element (PRE) which is required for the efficient nuclear export of viral mRNAs [16]. While no viral proteins have been reported to bind to the PRE, a number of cellular factors have, including La, glyceraldehyde-3-phosphate dehydrogenase, polypyrimidine tract binding protein 1, and members of the TREX mRNA export complex [17-19].

In this study, we performed an unbiased functional genomic screen in a human hepatoma-derived cell line (HepG2 2.2.15) [20] and identified multiple host proteins needed for late stage HBV replication. Subsequent experiments focusing on ZCCHC14 showed that the zinc knuckle protein is needed for HBV transcription and genome maintenance in both HBV cell culture systems and primary human hepatocytes (PHHs).

METHODS

Cell culture, virus and siRNA transfections

HepG2/HepG2.2.15/HEK-293T, HepAd38 and H25B10 hybridoma cells were cultured in

Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12 and RPMI 1640 media (Sigma), respectively. All media were supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 U/mL penicillin/streptomycin (Life Technologies). HepAd38 media was supplemented with tetracycline 0.3 µg/mL.

Generation of HepG2-NTCP cells

The retroviral expression vector, pQCXIH-NTCP-myc, was generated by amplifying the NTCP cDNA and cloning into pQCXIH (Clontech). pQCXIH+NTCP-myc was packaged in HEK-293T for retrovirus production. HepG2-NTCP cells were generated by transduction and selection with hygromycin 50 µg/mL. HBV particles were generated from HepAd38 cultured in media without tetracycline. Supernatants were supplemented with 8% PEG8000 (Sigma), precipitated overnight at 4 °C, centrifuged at 3500 g and resuspended in media. HBV infection of HepG2-NTCP cells was performed for 16 h with 4000 HBV genome equivalents/cell diluted in media, containing 4% PEG8000. Subsequently, cells were grown in media supplemented with 2.5% dimethyl sulfoxide (DMSO). Viral parameters were assessed at the time indicated for each experiment. In HepG2-NTCP, siRNA reverse transfection was performed 72 hours prior HBV infection, using siRNAs at the final concentration of 50 nM and RNAiMax (0.394% final v/v). PHH, purchased by Phoenix Bio Pharmaceutical (Japan), were shipped pre-plated in 24-well cell culture plates (Nunc) at 4×10^5 cells/well. PHH were infected with 25 HBV genome equivalents/cell for 24 hours. At day 4 and 7 post HBV infection, PHH were transfected with 25 nM siRNAs and DharmaFECT 4™ (Dharmacon, GE Life Sciences). In PHH, HBV parameters were analyzed 10 days post HBV infection.

siRNA screen

Cells were transfected in an arrayed format using a pooled version of the Ambion Silencer Select whole genome siRNA library (Life Technologies). The screen was performed in triplicate in 384-well plates (Corning #3712). 24 hours prior siRNA-transfection, HepG2.2.15 cells were passaged and replated at ~50% confluency in antibiotic-free DMEM containing 15% FBS and 10 mM

glutamine. On the day of transfection, Opti-MEM and RNAiMAX (0.394% final v/v) were aliquoted in order to obtain a siRNAs' concentration of 50 nM. A non-targeting (NT2, Dharmacon), HBV-targeting (HBV2) and Kif11-targeting siRNAs were included on every plate as negative, positive, and transfection efficiency controls, respectively. Reverse siRNA transfection was performed with 3300 cells/well, resuspended in antibiotic-free DMEM containing 15% FBS and cells were analyzed after 144 h. Cells were then fixed with 4% formaldehyde/phosphate buffered saline (PBS), permeabilized with 0.2% Triton X-100/PBS, and stained for HBsAg expression using supernatant from the H25B10 hybridoma and for DNA using Hoechst stain. Cells were imaged for percent HBsAg-positive and cell number using an ImageXpress Micro-XL (Molecular Devices) automated microscope (one 4X image per well, two wavelengths, FITC and DAPI) and analyzed using MetaXpress (Molecular Devices) cell scoring software. The percent HBsAg and cell number for each well was normalized to the plate mean. Pools whose transfection resulted in a >50% decrease without causing more than a 45% drop in cell number had their component siRNAs retested singly as above.

Microarray

Total RNA was isolated from HepG2 2.2.15 cells using an RNeasy kit (Qiagen), processed, and hybridized to an Affymetrix human GeneChip ST 2.0 microarray.

ELISA and cell viability

HBV HBsAg and HBeAg levels were measured in cell supernatant using CLIA ELISA Kits (Autobio Diagnostic), according to the manufacturer's protocol. After removal of the supernatant, cells were incubated with media additionally containing "Cell Counting Kit – 8" (Sigma-Aldrich) for one hour and supernatants were measured (EnVision®, PerkinElmer).

Immunoblotting

Whole cell lysates were prepared with Laemmli buffer. Samples were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to polyvinylidene fluoride (PVDF) membrane and blocking with

2% non-fat milk BRCA1 and Ran were detected using BRCA1 (Santa Cruz Biotechnology, C-20) and ARAN1(Sigma R4777) primary antibodies.

HBV DNA and cccDNA measurement

Standard curves for HBV DNA and cccDNA measurement were generated as following: pBR322 HBV (*ayw* genotype [21]) was digested with EcoRI and HBV genome was gel purified and quantified. HBV genome standards ranging between 10^6 and 1 copies of HBV genome were used as templates for cccDNA qPCR (cccDNA_FW: 5'-CGTCTGTGCCTTCTCATCTGC-3'; cccDNA_REV: 5'-GCACAGCTTGGAGGCTTGAA-3'; cccDNA_probe (MGB): 5'-CGTGAACGCCACCGA-3') and HBV DNA (HBV_FW: 5'-TCCTCTTCATCTGCTGCTATG-3'; HBV_REV: 5'-CGTGCTGGTGGTTGAGGATCCT-5'; HBV_probe: 5'-TGTTGGTTCTTCTGGACTA-3'). Standard curves for the number of haploid cellular genomes (that served as normalization for cells number) were prepared with genomic DNA standards and amplified with a TaqMan Gene Expression Assay (cat. n. Hs02786624_g1, ThermoFisher Scientific). Genomic DNA samples were subjected to qPCR with the previously indicated combination of primers and probes. Copy numbers were extrapolated from standard curves. Normalization was performed first for the number of haploid genomes and then for the non-targeting (NT) control samples. qPCR was performed with a LightCycler 480 II.

Reverse Transcription and qPCR

RNA was extracted from HepG2.2.15 and HepG2-NTCP cells with RNeasy Mini Kit (Qiagen) and RT-qPCR was performed on a LightCycler 480 II using a QuantiTect Multiplex RT-PCR kit (Qiagen). The following primer/probe sets were used: GAPDH (Hs0275899_g1), SIRT2 (Hs00247263_m1), SMARCE1 (Hs00705034_s1), YWHAH (Hs00607046_m1), and ZCCHC14 (Hs00296622_m1). PHH were lysed using the MagNA Pure "96 Cellular RNA Large Volume Kit" (Roche). For the RT-qPCR reaction an AB7900 HT sequence detection system (Applied Biosystems), the TaqMan[®] Gene Expression Master Mix (ThermoFisher Scientific) and pre-designed ZCCHC14-specific primer (Hs00296622_m1) were used. Samples were normalized to

β -actin measured with a TaqMan Expression Assay (Hs01060665_g1, ThermoFisher).

In HepG2-NTCP cells, HBV total RNA and 3.5 kb HBV RNA were detected with iQ SYBR Green Supermix reagent according to the manufacturer protocol, using the primers in [4] and GAPDH primers GAPDH_FW: 5'-TGCACCACCAACTGCTTA-3', GAPDH_REV: 5'-GGATGCAGGGATGATGTT-3'. Relative expression was calculated with the $2^{-ddC(t)}$ method.

Northern blot

To examine the effect that depletion of selected HBV-HF had on HBV transcript and cccDNA accumulation, 6.25×10^5 HepG2 2.2.15 cells per well in 6-well plates were transfected with the NT2 or HBV2 siRNAs or siRNAs (Life Technologies) against BRCA1 (s224683), SIRT2 (s22706), SMARCE1 (s13160), YWHAH (s14968), or ZCCHC14 (s23202) as above. At 6 d post transfection, total RNA was isolated from cells using an RNeasy kit and quantified using a Nanodrop (ThermoFisher). 5 μ g total RNA was denatured in formamide/formaldehyde/MOPS at 55 °C for 30 min and then resolved on a 1% formaldehyde/MOPS/agarose gel, and transferred to a Hybond-N membrane overnight. After transfer, the membrane was stained with methylene blue to check transfer efficiency and to visualize 28S and 18S RNA. A region of HBV corresponding to coordinates -260 to 1991 was amplified using Phusion polymerase and the primers 5'-aaaaaaAAGCTTCCCGGGcaaacaccgcaatccagatt-3' and 5'-aaaaaaGCGGCCGCGCCATATGgatctcgtactgaaggaaagaagt-3' and subsequently used to produce a digoxigenin-labeled probe using the DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer's instructions. The membrane was probed according to "RNA: A Laboratory Manual" and probe detected with anti-DIG antibody according to the High Prime manual. Labeled RNA was visualized using X-ray film.

RESULTS

Whole genome RNAi screen for HBV-HFs

To find host factors that modulate late stage HBV replication, we carried out an RNAi screen using

a human hepatoma cell line, HepG2 2.2.15, which contains an integrated array of HBV genomes and constitutively produces infectious HBV [20]. Given these characteristics, we reasoned that host factors required for the later stages of HBV replication could be identified in a genetic screen employing this cell line. We optimized the screen platform by reverse transfecting cells with a positive control siRNA (HBV2) that targets all four of the HBV mRNAs. After 144 h post-transfection, we determined the levels of cell-surface associated HBsAg using immunofluorescence (IF) microscopy (Figure 1A, B). This assay was then used to screen a whole-genome siRNA library, Silencer Select, in which 21,584 genes are targeted by three siRNAs per gene, each combined into a pool (Figure 1C). We selected 694 genes as candidates for validation by virtue of their respective siRNA pools decreasing HBsAg immunostaining to $\leq 50\%$ of the plate mean while maintaining cell number at $\geq 45\%$ of the plate mean. Notably, the screen detected the known HBV host-factors (HBV-HF), HNF1 and PRMT1, as modulating HBsAg expression (Figure 1C). The screen identified several pathways and complexes whose components scored in the primary screen dataset (Figure 1D). In addition to host factors required for HBsAg expression (dependency factors [DFs]), we also detected complexes and pathways whose loss enhanced viral infection (competitive factors [CFs]).

Gene expression filtering of HBV-HF candidates

While siRNA screens are useful for discovering human-virus interactions, they also harbor false positives or off-target-effects (OTEs) [22, 23]. We used gene expression filtering to decrease the number of OTEs selected for in our validation screen. To this end, RNA was collected from HepG2 2.2.15 cells and mRNA expression levels of individual genes were determined by microarray (Figure 1E). Using these expression data, we excluded all candidate genes whose expression signal fell below the intronic mean.

We then used the following criteria: strength of phenotype, novelty, and informatics, to select siRNA pools for the validation round, which involves testing each of the three individual

siRNAs from the chosen pools. Of those tested, 260 pools were found to confirm with one siRNA and 69 candidates confirmed with two or more siRNAs using the same criteria as in the primary screen (Table S1). Pools with two or more siRNAs that scored are higher confidence because such results are consistent with the intended target's depletion.

From this set of higher confidence candidates, we selected five for which multiple orthologous siRNAs produced the largest average decrease in HBsAg surface immunostaining: the E3 ubiquitin ligase, BRCA1; the deacetylase, SIRT2; the chromatin modifier, SMARCE1; the 14-3-3 family member, YWHAH, and the zinc-knuckle protein, ZCCHC14. Depletion of these genes was retested in a 96-well format and evaluated for the effect on HBsAg expression (Figure 2A, B). All candidates confirmed the initial host dependency factor phenotype with multiple siRNAs. Strikingly, introduction of siRNAs against ZCCHC14 resulted in a near total loss in both HBsAg staining and secretion – being even lower than that of the siRNA that targets viral transcripts directly. siRNAs against these five genes were then tested for target depletion and the phenotypes were found to be commensurate with the levels of expression (Figure 2C, D).

Role of HBV-HFs in viral transcription

We next examined how the downregulation of these factors affected viral transcription. HepG2 2.2.15 cells were transfected with the most potent respective siRNAs against BRCA1, SIRT2, SMARCE1, YWHAH, or ZCCHC14 (Figure 3A). After 144-h post-transfection, total RNA was isolated and viral RNA was examined by Northern blotting. As expected, the anti-HBV siRNA produced a loss of all HBV RNA species. siRNAs against BRCA1, SIRT2, and YWHAH also resulted in a decrease in one or more viral transcripts. Interestingly, loss of BRCA1 appeared to decrease the levels of the pgRNA/preC vRNA, while leaving the X vRNA unchanged. In contrast, the depletion of SIRT2 and YWHAH resulted in a pronounced decrease in just the levels of the X vRNA. Much like its effect on HBsAg production, depletion of ZCCHC14 had the strongest effect on viral transcription, causing

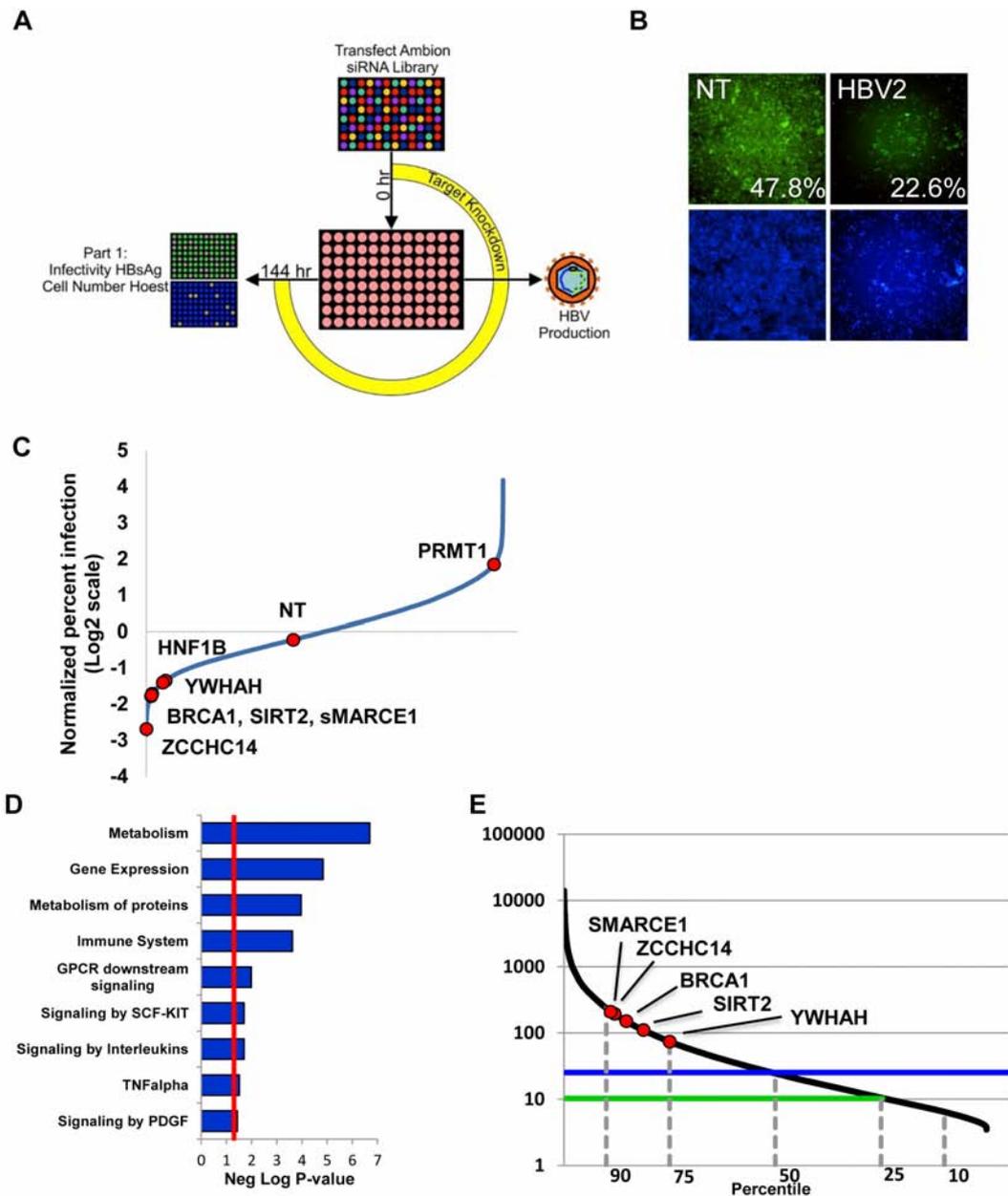


Figure 1. A high-throughput siRNA screen identifies multiple HBV-HFs. A) schematic of the HBV-HF screen, where HBsAg expression is modulated by siRNA-mediated knockdown of host proteins. Alterations are detected using immunofluorescence (IF) imaging. B) An example of cells transfected with a non-targeting (NT) and an HBV-targeting siRNA are shown. HBsAg is shown in green and cellular DNA (as detected by Hoechst dye) in blue. C) Arrayed data from the whole-genome siRNA screen for HBsAg expression in the HepG2 2.2.15 cells (normalized percent infection Log₂ scale). NT is the negative control siRNA and HBV is a siRNA against a region shared amongst the HBV transcripts. HNF1B and PRMT1 are factors known to facilitate or restrict, respectively, HBV replication. BRCA1, SIRT2, SMARCE1, YWHAH, and ZCCHC14 are factors found in the screen to be required for HBV replication. D) Enriched Gene Ontology terms amongst the factors identified in the primary siRNA screen. E) Ranked gene expression levels based on data from experiments using an Affymetrix human GeneChip ST 2.0 microarray hybridized with cDNA from HepG2 2.2.15 cells (represents the average of three independent chip datasets). The relative expression levels of the indicated high confidence HBV-HFs are noted, with the intronic mean serving as a cutoff (blue line).

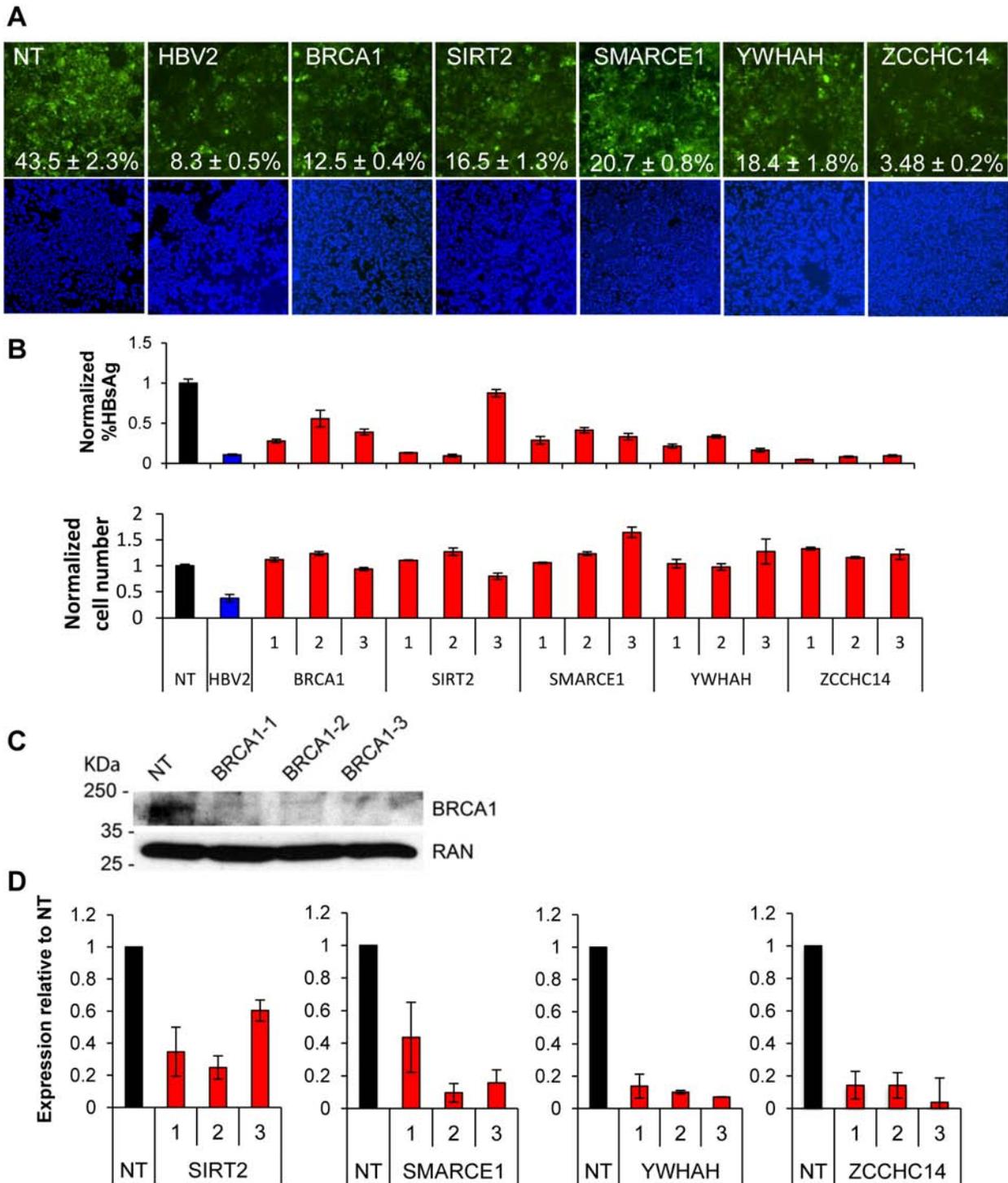


Figure 2. Validation of candidate HBV-HFs. A) HepG2 2.2.15 cells were transfected with individual siRNAs against candidate HBV-HFs and then examined for their ability to decrease HBsAg staining by immunofluorescence. HBsAg is shown in green and cellular DNA in blue. B) Quantitation of HBsAg staining (top panel) is expressed as percentage of HBsAg positive cells relative to NT control. Cell viability (bottom) is shown as cell number relative to NT. C) Representative immunoblot of three independent experiments for BRAC1 (top), RAN is the loading control (bottom). D) qPCR for SIRT1, SMARCE1, YWHAH and ZCCHC14 (n = 3); values are mean ± SD normalized to NT.

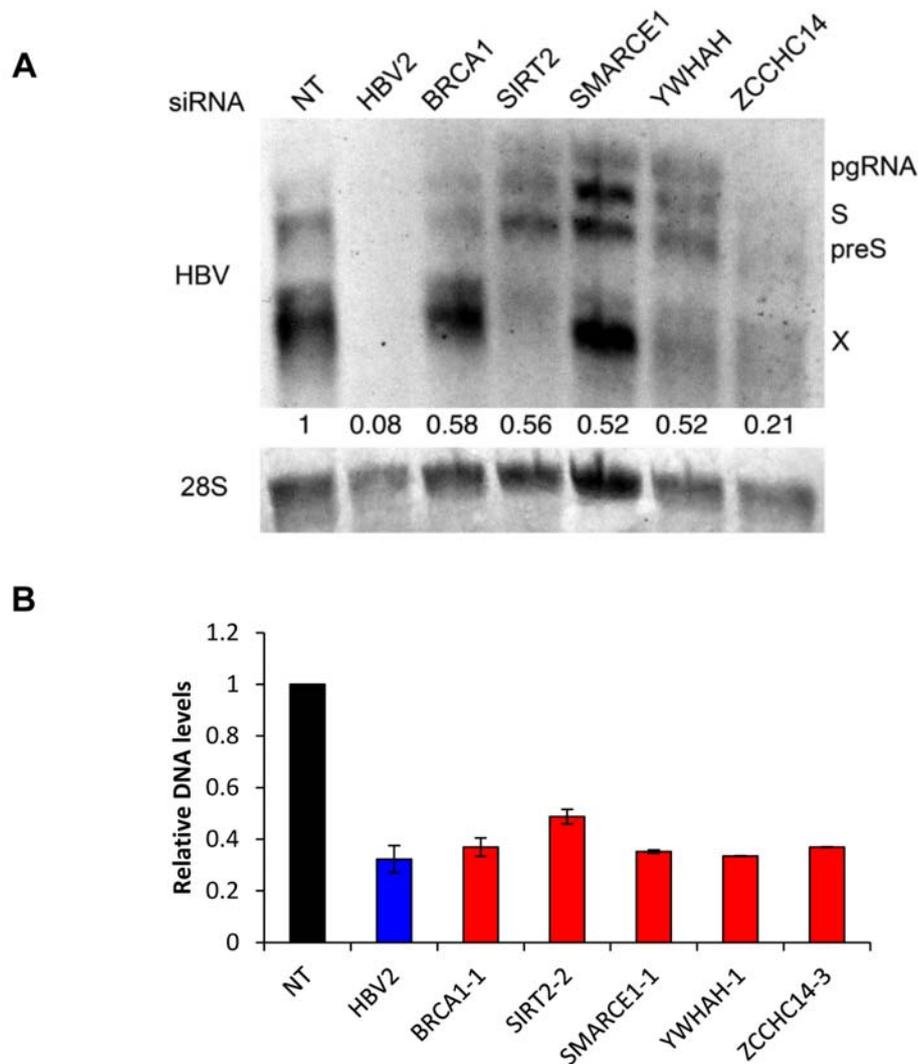


Figure 3. Knockdown of multiple HBV-HFs leads to a decrease in viral transcription and HBV DNA level. A) HepG2 2.2.15 cells were transfected with siRNAs against HBV-HFs and then evaluated by Northern blot for accumulation of viral transcripts using a digoxigenin-labeled DNA probe 5 days post transfection. Quantification of Northern blot represent the levels of total HBV RNA present, relative to the siNT-transfected lane and normalized to 28S RNA and are representative of three independent experiments. B) qPCR for HBV DNA, $n = 2$; error bars +/- SD.

a marked loss of all vRNA species, mirroring the result of the HBV2 siRNA which directly targets the viral RNAs. These results indicate that the selected HBV-HFs, with the exception of SMARCE1, are necessary for the transcription of one or more viral RNAs.

HBV's pgRNA transcript encodes for the polymerase and core proteins and serves as a template for rcDNA. We tested the effect of siRNA-mediated depletion of the selected HBV-HFs on HBV DNA levels in HepG2.2.15 cells

using qPCR as the viral DNA in these cells is primarily comprised of rcDNA. RNAi silencing produced a decrease in HBV DNA levels for each factor tested, consistent with the observed effect on pgRNA (Figure 3B).

ZCCHC14 is required in a fully infectious HBV tissue culture system

We next investigated ZCCHC14's role using a fully infectious HBV cell culture system [4]. To this end, HepG2 cells were stably transduced with

NTCP. After selection, we confirmed NTCP expression and the permissivity of the resultant HepG2-NTCP cells to HBV infection based on the intracellular expression of HBsAg and the secretion of HBeAg after viral challenge (Figure S1A, B). HepG2-NTCP cells were then transfected with 9 multiple siRNAs against ZCCHC14, and three days later, infected with HBV. Seven days post-infection, cells were assayed for HBsAg by IF microscopy (Figure 4A, B). The depletion of ZCCHC14 mRNA was confirmed by quantitative real-time PCR (qPCR, Figure 4C). For each of the orthologous siRNAs tested, ZCCHC14 diminishment decreased HBV replication. Consistent with the data using HepG2 2.2.15, the targeting of ZCCHC14 in HBV-infected HepG2-NTCP lowered the levels of both HBV mRNA and HBV DNA content (Figure 4D, E). cccDNA was still detectable in HepG2-NTCP cells but its levels were lowered with ZCCHC14 silencing (Figure 4E).

Given these data using transformed cells, we tested ZCCHC14's role in HBV replication in PHH. To test whether depletion of ZCCHC14 can decrease an established infection, we first infected PHH for ten days with HBV, then decreased ZCCHC14 levels by transfecting a pool of 4 siRNAs into the infected primary cells. The post-infection reduction in ZCCHC14 resulted in a decrease of HBV core mRNA, as visualized by fluorescence in-situ hybridization (Figure 5A), in comparison with PHH transfected with a pool of negative control siRNAs. An RNAi-mediated decrease of ZCCHC14 was confirmed by qPCR (Figure 5C). Furthermore, we observed a significant decrease in the levels of secreted HBSAg and HBeAg in the supernatant of PHH silenced for ZCCHC14 (Figure 5B). Finally, the HBV DNA content in PHH supernatant, which accounts for the secreted viral particle, was also significantly affected by the silencing of ZCCHC14 (Figure 5D). We conclude that ZCCHC14 is required for HBV replication in PHH.

DISCUSSION

Genetic screens are useful for identifying host factors that modulate viral replication. Therapeutic targeting of viral host factors has the potential to prevent, treat and cure viral infections. To discover HBV-HFs, we undertook a whole genome RNAi

screen using the constitutively HBV-infected HepG2 2.2.15 cell line. The screen identified sixty-nine high confidence HBV-HFs that confirmed with multiple independent siRNAs. Based on i) their strength of phenotype, ii) the number of independent siRNAs that confirmed in the validation round, and iii) their novelty in HBV replication, we chose five of these genes for further study. We found that the HBV-HFs, BRCA1, SIRT2, YWHAH, and ZCCHC14 are all required for efficient HBV transcription. Given that the most proximal step in the HBV lifecycle is transcription of viral mRNA from integrated proviruses, our finding that these HBV-HFs are all important for viral transcription suggests that the strongest hits act very early in the viral lifecycle; this notion is also consistent with our previous viral host factor screening studies. Since a limitation of this study is its reliance on the HepG2 2.2.15 system, it is important to note that these select HBV-HFs decreased the replication of HBV in a fully infectious system using HepG2-NTCP cells.

YWHAH is a member of the 14-3-3 family, a set of widely expressed phospho-serine/threonine-binding proteins that can act as molecular chaperones, scaffolds, or adaptors [22-24]. The human genome encodes seven different 14-3-3 proteins: YWHAB, YWHAE, YWHAG, YWHAH, YWHAQ, YWHAS, and YWHAZ [25]. These proteins share between 60-90% amino acid identity, but vary in the specific proteins with which they interact and the pathways in which they function [26]. Although 14-3-3 proteins have no known intrinsic activity of their own, it has been proposed that they alter the functions of their interacting proteins by influencing their localization, preventing dephosphorylation, or altering their ability to associate with targets [24]. While not an absolute rule, 14-3-3 proteins generally interact with their partners in a phosphorylation-dependent manner. Additionally, three 14-3-3 interaction sequences have been described, including mode I RXX(pS/pT)XP, mode II LX(R/K)SX(pS/pT)XP, and mode III pS/pTXX motifs [25]. Interestingly, both HBV pol and HBx contain one more of these motifs. Furthermore, HBx was previously shown to colocalize with a complex containing YWHAB and the potential 14-3-3 binding motif in HBx was found to be necessary for activation of stress-

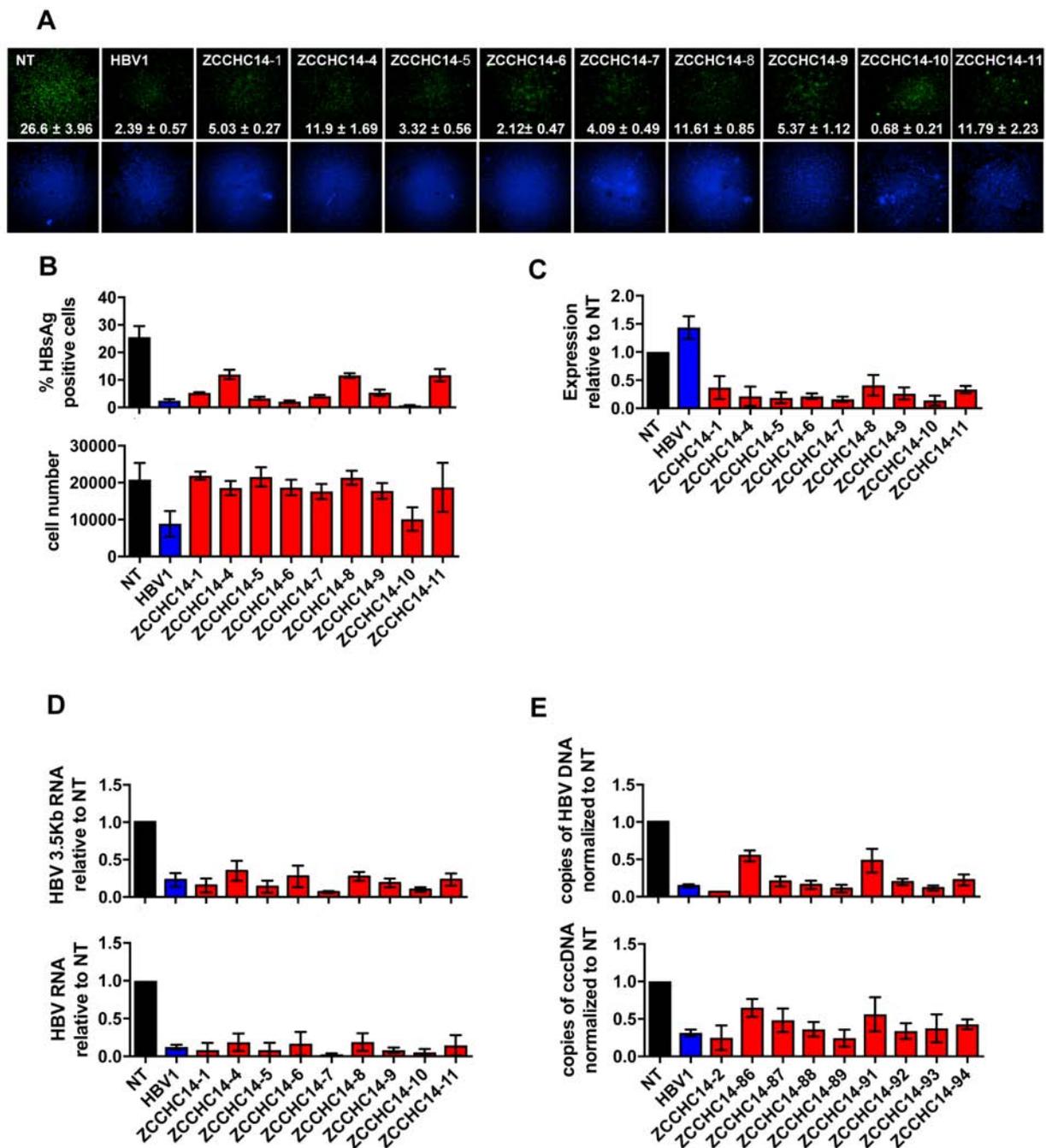


Figure 4. ZCCHC14 knockdown in HBV-infected HepG2-NTCP cells suppresses HBV replication. HepG2-NTCP cells were transfected either with NT siRNA, a siRNA that targets a region shared amongst the HBV transcripts (HBV1) or nine siRNAs targeting ZCCHC14 coding sequence. 72 hours post transfection, cells were infected with HBV for 16 h. 7 days post infection HBsAg staining, ZCCHC14 mRNA, HBV RNAs, total HBV DNA and cccDNA measurements were performed. A) Cells were stained for HBsAg (green) and cellular DNA (blue), representative images of two independent experiments. B) Quantitation of the percentage of HBsAg positive cell (top) and cell number (bottom) is reported; $n = 3$, values are mean \pm SD. C) Expression of ZCCHC14 was assessed by qPCR, $n = 3$; values are mean \pm SD, normalized to NT. D) Expression of HBV 3.5Kb RNA (top) and total HBV RNA species (bottom) was performed by qPCR, $n = 3$; values are mean \pm SD. E) Copy number of HBV DNA (top) and cccDNA (bottom) were assessed by qPCR, $n = 3$; value are mean \pm SD normalized to NT.

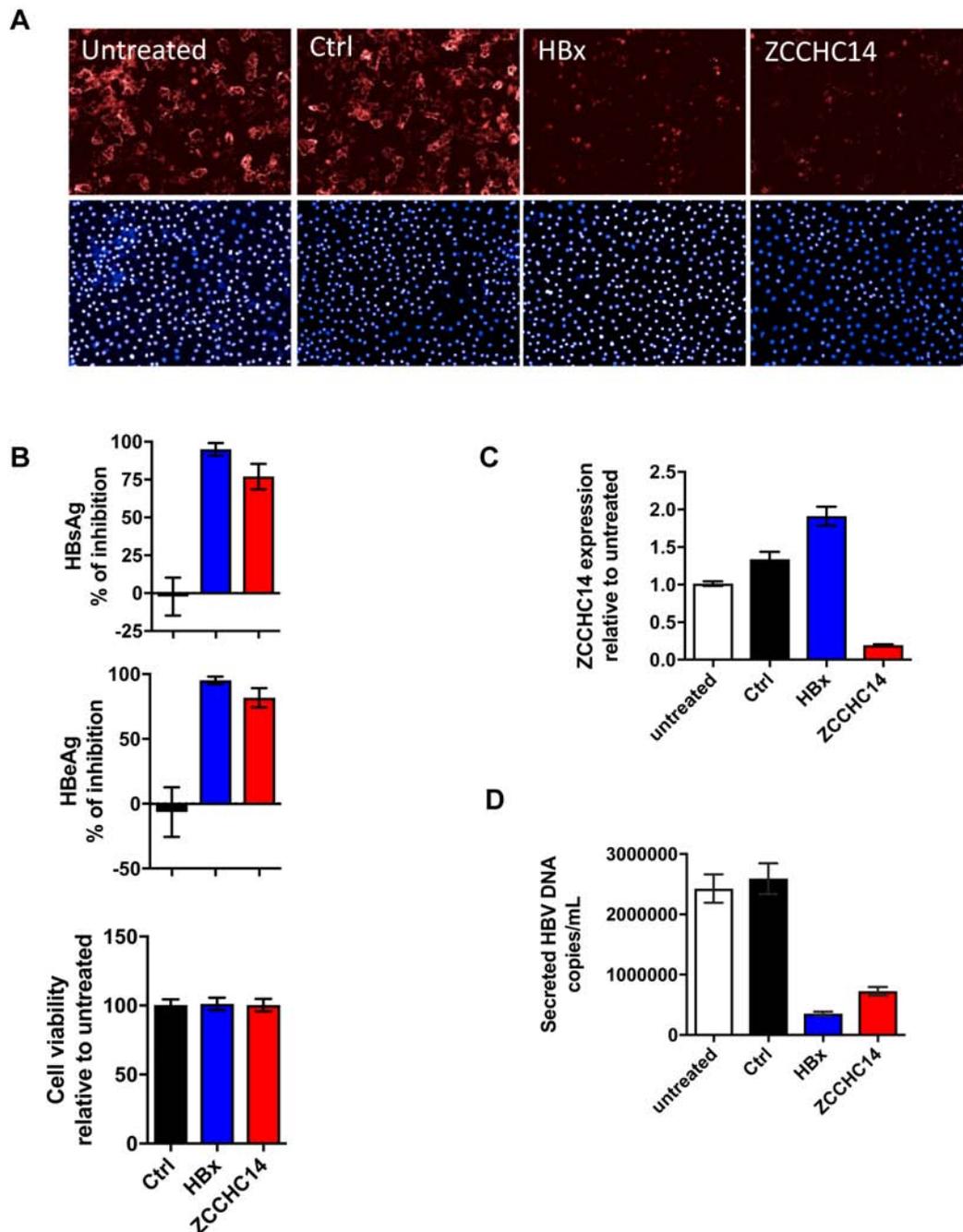


Figure 5. Knockdown of ZCCHC14 post HBV infection leads to suppression of HBV replication in primary human hepatocytes (PHH). Purified PHH were left uninfected or infected with HBV and treated with siRNA against either HBx (siHBx), ZCCHC14 (siZCCHC14, a pool of 4) or siRNA control (siCtrl, a pool of 4) at day 4 and 7 post HBV infection. Cells were harvested on day 10 post-HBV infection. A) Fluorescence microscopy was performed to assess HBV infection levels: HBV core mRNA was visualized using ViewRNA[®] *In Situ* Hybridization (red), cell nuclei were stained with DAPI (blue). B) Levels of HBsAg and HBeAg (top and middle) were assessed by ELISA; viability of cells (bottom) was measured using the CCK8 viability assay and normalized to the non-treated control, n = 3; values are mean \pm SD. C) At day 10 post HBV infection, expression of ZCCHC14 was assessed by qPCR. Values were normalized for the non-treated condition, n = 3; values are mean \pm SEM. D) Copy number of secreted HBV DNA was assessed by ELISA on cell supernatant, n = 3; values are mean \pm SEM.

associated kinases and protection from apoptosis [27]; however, no cytotoxicity was observed upon YWHAH depletion, arguing that these two 14-3-3 proteins play different roles in HBV replication. Our data, instead, reveal that YWHAH is required for HBV transcription. Interestingly, 14-3-3 proteins have been implicated in several of the same gluconeogenic pathways that activate HBV transcription. For instance, YWHAH has been shown to interact with GR, stabilizing the protein and enhancing GR-mediated transcription [28]. We hypothesize that YWHAH regulates a specific cellular and/or viral protein that is required for viral transcription.

CONCLUSION

ZCCHC14 was one of the strongest hits found in the HBV-HF screen. While next to nothing is known about this protein, its CCHC-type zinc knuckle domain (also known as the Gag knuckle) places ZCCHC14 in a family of proteins which bind and modify RNAs. The prototypical zinc-knuckle proteins, the retroviral gag proteins, bind to and direct the packaging of retroviral RNAs *via* the actions of their knuckles [29]. Perhaps the best studied mammalian CCHC-protein, Lin28A (ZCCHC1), binds to cellular mRNAs in the nucleus, translocates with them to the cytoplasm, and appears to enhance their translation by promoting their localization to P-bodies [30]. Due to the strong reliance of HBV transcription on ZCCHC14 and the likelihood that ZCCHC14 binds RNA, we speculate that ZCCHC14 may interact with HBV mRNAs, perhaps through their shared posttranscriptional regulatory element. Consistent with this notion, ZCCHC14 was strongly required for HBV replication in HepG2-

NTCP cells and PHH cells. Interestingly, the HepG2-NTCP cell studies showed that upon ZCCHC14 silencing, the impairment of pgRNA transcription was accompanied by a significant reduction of the total HBV DNA, which mainly is comprised of rcDNA. cccDNA content was also affected by ZCCHC14 depletion, potentially due to the impaired recycling of rcDNA to the nucleus for the maintenance of the cccDNA pool. In conclusion, our whole genome siRNA screen using HepG2 2.2.15 cells identified multiple high confidence HBV-HFs, including the zinc knuckle protein, ZCCHC14. Additional studies will now be needed to more fully elucidate the actions of ZCCHC14 and the other high confidence HBV-HFs in viral replication.

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AUTHOR CONTRIBUTIONS

MCS, GS, AMA and ALB set up and carried out the primary and secondary siRNA screen, MCS, JMPo, JMPe, MK, WM, and ALB designed and performed follow up and validation experiments, HM and HJ designed and carried out HepaRG and primary hepatocyte experiments. MCS, HM, HJ, WM and ALB wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

MCS and ALB have filed for patent protection for host factors recovered in this screen.

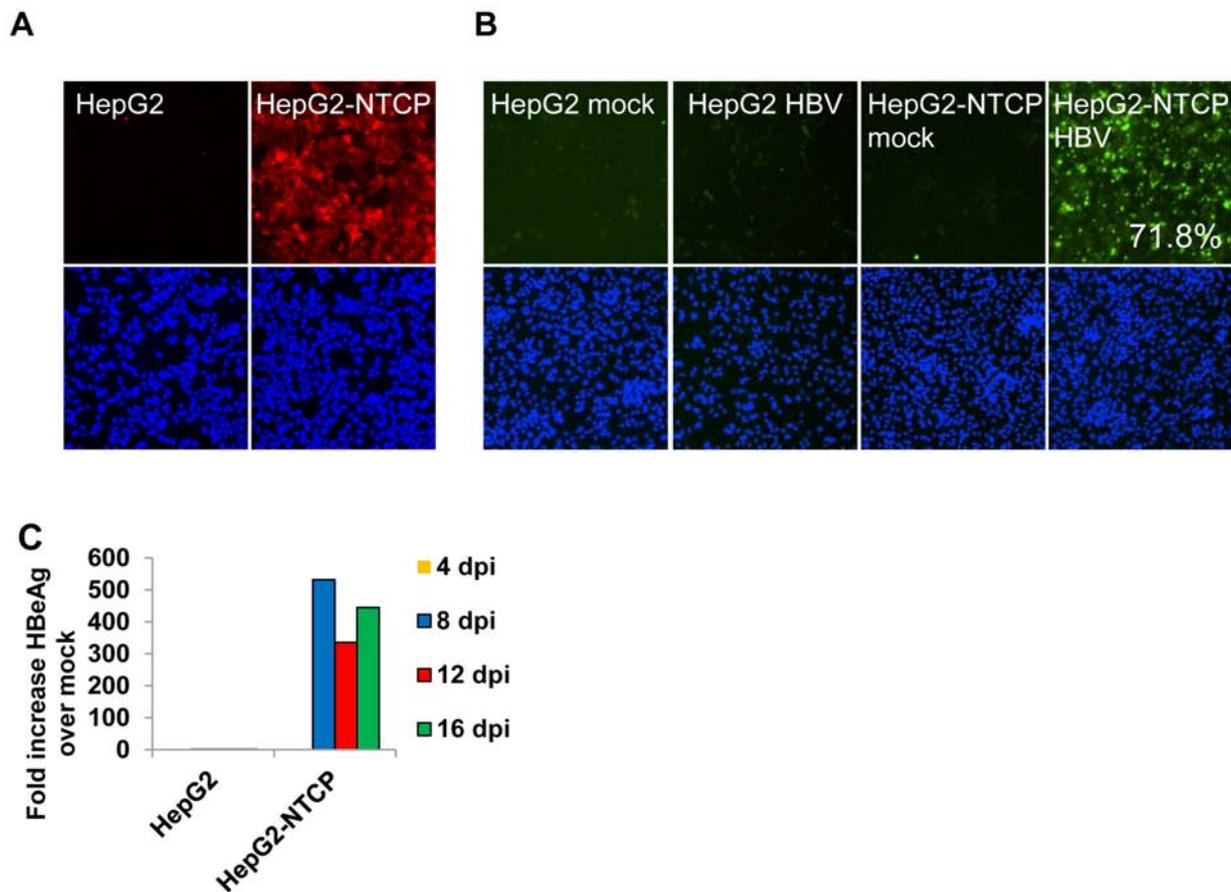
SUPPLEMENTARY MATERIALS

Supplemental Table 1. siRNA sequences used in this manuscript. Sequences and catalog numbers of all siRNAs used for validation of HBV-HFs.

List of siRNAs employed in the validation of HBV-HFs				
siRNA	Company	Reference	Sense 5' -> 3'	Antisense 5' -> 3'
NT2	Ambion	CTM-40421	uaaggcuaugaagagauacuu	guaucucucauagccuuuuu
HBV1	Ambion	RD-08986	uacuagugccauuuguuacuu	ugaacaaauggcacuaguauu

Supplemental Table 1 continued..

List of siRNAs employed in the validation of HBV-HFs				
siRNA	Company	Reference	Sense 5 '-> 3'	Antisense 5 '-> 3'
HBV2	Ambion	RD-08660	gcacucgcucaccucuguu	cagaggugaagcgaagugcuu
siKIF11	Ambion	s7904	ccacgucuccuaucauatt	uuugaugaaggguacguggaa
siBRAC1-1	Ambion	s224683	gaccagucuaauuaaaagaatt	uucuuuaauagacugggucac
siBRAC1-2	Ambion	s458	cagcuacccuuccaucauatt	uauauggaaggguagcugt
siBRAC1-3	Ambion	s459	caugcaacauaaccugauatt	uaucaagguuauugucauggt
siSIRT2-1	Ambion	s22706	gcccaagugugaagacugutt	acagucucacacucugggcgt
siSIRT2-2	Ambion	s22707	agaaacaucgggaaccuutt	aagggucgggauguuucutg
siSIRT2-3	Ambion	s22708	gcucaucaacaaggaggaatt	uuucuccuuguugaugagcag
siSMARCE-1	Ambion	s13060	gcaagaaaagauagaguacatt	uguacucuaucuuuucugctt
siSMARCE-2	Ambion	s13061	guaccuugcuuacauaaaatt	auuuauugaagcaagguacgc
siSMARCE-3	Ambion	s13062	gaccuaaaguuguggagatt	ucuccacaacuuuaggucag
siYWHAH-1	Ambion	S14967	caagguguuuuaccugaatt	uuucaggguaaaaacaccuuggt
siYWHAH-2	Ambion	S14968	cacuaaacgaggauucuaatt	uagaauccucguuuagugt
siYWHAH-3	Ambion	S14969	gaaugaaccucucuccaatt	auuggagagagguucauucag
siZCCHC14-1	Ambion	S23202	gucugauucuaauaacatt	uguuauugaagaucagacca
siZCCHC14-2	Ambion	S23203	gcauuuuauuggagcgaatt	uucgcuccacauaaaugcgt
siZCCHC14-3	Ambion	s23204	ccuucucacguguugaaaatt	uuuuaacacgugagaggta
siZCCHC14-4	Ambion	s529886	gaauuuuugagucucuutt	aagagacuaauuuuauucag
siZCCHC14-5	Ambion	S529887	gcaagugagugiugaaaatt	uuuuaacacucacuucugctg
siZCCHC14-6	Ambion	S529888	gcagcuucagaguccaagutt	acuuggacucugaagcugctg
siZCCHC14-7	Ambion	S529889	gugacggaauuuuuuuaatt	uugaaauuuuuccgucactt
siZCCHC14-8	Ambion	S529891	caauccuccuuucuaaatt	uuuagaaaggaggauugcc
siZCCHC14-9	Ambion	S529892	gaggucuugggucugauutt	aaucagaccacaagaccucaa
siZCCHC14-10	Ambion	S529893	agaccugaagggaauuacatt	ugauuuuuuuccagguuat
siZCCHC14-11	Ambion	S529894	caauaacaucaguaaccaatt	uugguuacugauguuuuugaa
siZCCHC14 (pool)	Dharmacon	#L-014086-01		
siCnt (pool)	Dharmacon	#D-001810-10		
siHBVx	Dharmacon	custom made	gcacucgcucaccucuguu	



Supplemental Figure 1. HBV-HFs are required by HBV in the fully infectious system. A) HepG2 cells were stably transduced with an empty lentiviral vector or one that encodes the HBV receptor, NTCP and examined for NTCP expression by IF. B) Vector and NTCP-transduced HepG2 cells were then either mock infected or infected with HBV+ serum. Cells were fixed and stained for HBsAg at 16 dpi and supernatant from 4, 8, 12, and 16 dpi was analyzed for HBeAg by ELISA. C) HepG2-NTCP cells were then transfected with an NT siRNA, an siRNA targeting HBV, or one targeting an HBV-HF. Three days later, the cells were infected with HBV+ serum. At 7 dpi, cells were fixed and stained for HBsAg. n=2, error bars +/- SD.

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