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Supplemental information

Mechanism of action of hepatitis B virus S antigen transport-inhibiting oligonucleotide polymer, STOPS, molecules

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SUPPLEMENTAL MATERIALS

Results

Functional analysis of STOPS action. STOPS might inhibit HBV infection by acting inside or outside of the cell. To elucidate this, we either added ALG-10000 to the media of HepG2.2.15 cells or used lipofectamine RNAiMax to transfect STOPS into cells. HBsAg levels were reduced only when ALG-10000 was transfected into cells; no reduction in HBsAg was observed even when a ten-fold higher concentration of ALG-10000 was added to the cell culture media (Figure S1A). Thus, STOPS need to function within cells to inhibit HBsAg.

The intracellular location of the transfected ALG-10000 was examined using a Cy5-labeled ALG-10000 molecule. After transfection Cy5-ALG-10000 appeared mostly in the cell cytoplasm as somewhat discrete structures (Figure S1B). HBsAg also localizes in discrete structures in HepG2.2.15 cells.[1] However, quantification of the fluorescence of HBsAg and Cy5-ALG-10000 revealed that only between 10-14% of Cy5-ALG-10000 co-localized with HBsAg 24 h after transfection (Figure S1C). These results suggest that it is unlikely that ALG-10000 directly binds to HBsAg.

We examined whether ALG-10000 could activate two intracellular innate immune receptors that bind nucleic acids: Toll-like receptor 3 and RIG-I. Human embryonic kidney (HEK) 293 cells at 4 x 10^4 cells /mL were transfected with three plasmids, one to express the receptor, a second that contains a firefly luciferase driven by the NF-Kβ transcription factor (that responds to TLR3 and RIG-I), and a third to express a constitutive *Renilla* luciferase.[2] The cells were grown for 24 h prior to super-transfection with ALG-10000 to a 50 nM final concentration. 24 h later the luciferase signal and the constitutive *Renilla* luciferase were quantified using the Dual-Glo Luciferase Assay System (Promega) and a plate reader (Biotek Inc). ALG-10000 failed to activate TLR3 or RIG-I

signaling, while poly(I:C), a known agonist for both TLR3 and RIG-I, resulted in significant activation of the firefly luciferase (Figure S1D and S1E).

STOPS-mediated reduction of intracellular HBsAg occurs in a time-dependent manner. To determine whether ALG-10000 inhibits intracellular HBsAg, HepG2.2.15 cells were transfected with 8 nM ALG-10000 or 20 nM REP 2139, and the cells were lysed for Western blot analysis over several time points. Intracellular HBsAg was identified using an antibody that specifically recognizes all three forms of HBsAg (20-HR20, Fitzgeralds Industries). However, in the HepG2.2.15 cells, a spurious band recognized by the antibody co-migrated close to the Small HBsAg making it less accurate to quantify the Small HBsAg. Hence, the Large HBsAg is shown (Figure S2). The reduction of the Large HBsAg by ALG-10000 was detected at 65 h after transfection (not shown) but was more evident after 76 h and reached maximum levels at about 88 h (Figure S2). REP 2139, which is less effective in reducing extracellular HBsAg, was also less effective in reducing the levels of intracellular HBsAg levels. The timing for the reduction of intracellular HBsAg mirrors the inhibition of extracellular HBsAg (Fig. 2B), suggesting that STOPS acts on the intracellular HBsAg.

SiRNAs knock down both the target mRNA and protein. To confirm that the siRNAs can reduce their targets, we examined the amount of target RNAs present after siRNA treatment. HepG2.2.15 cells transfected with 10 nM of the siRNAs targeting GRP78, RPLP1 and RPLP2 for 72 h were lysed, and the RNA extracted in the plates and subjected to real-time RT-PCR. All three mRNAs were reduced by more than 80%.

We also examined the amounts of all five target proteins in cells transfected with siRNAs. HepG2.2.15 cells transfected for 72 h were lysed and the protein lysate subjected to Western blot

probed with antibodies specific to the target proteins. The amounts of the proteins present were quantified using ImagJ. All five target proteins were reduced by cognate siRNA. The knockdown of RPLP1 affected the amounts of RPLP2 and vice versa. This is expected, as the two proteins are known to stabilize each other.[3] Unexpectedly, however, the knockdown of RPLP1 and RPLP2 also resulted in an appreciable reduction in the levels of GRP78. These results suggest that RPLP1/2 could also interact with GRP78.

ALG-10000 can reduce the amount of SRSF1 in a concentration-dependent manner. HepG2.2.15 cells transfected with ALG-10000 exhibit reductions of the cellular proteins in a time-dependent manner. The reduction in the SRSF1 was also dependent on the concentration of ALG-10000 transfected (Figure S4). Notably, REP 2139 had less of an effect on the level of SRSF1.

Validation of GRP78's role in HBV replication. GRP78 is a member of the Hsp70 family of chaperone proteins that functions in the endoplasmic reticulum to bind client membrane proteins to mediate their folding or direct their degradation. GRP78 has been implicated for HBV replication.[4] To further confirm that GRP78 functions in HBV protein production/degradation, we tested known properties of GRP78. First, Hsp70-like chaperone proteins function with co-chaperones and co-chaperone DNAJB9 has been shown to interact with GRP78.[5] Knockdown of the DNAJB9 should also cause a reduction in HBsAg levels. SiRNA that knocked down DNAJB9 resulted in a reduction in the levels of extracellular HBsAg (Figure 5A). Knockdown of DNAJB12, a co-chaperone that does not interact with GRP78, did not reduce HBsAg levels. These results support the idea that GRP78 functions in HBV replication and HBsAg production/degradation.

Mycotoxin Deoxynivalenol (DN), is known to target GRP78.[6] If GRP78 functions in HBV HBsAg production, treating cells with DN should affect HBsAg production. Treatment with DN

at low micromolar concentrations exhibited no toxicity to HepG2.2.15 cells but resulted in a concentration-dependent reduction in the extracellular HBsAg (Figure S5B). Western blot analysis showed that the levels of Large HBsAg were also reduced (Figure S5C). The results with DNAJB9 and DN confirm that GRP78 functions in HBsAg production.

STOPS can reduce the levels of the HBV polymerase. STOPS ALG-10000 and the knockdown of the five host factors reduced the HBV polymerase amounts in HepG2.2.15 cells (Fig. S2D). To confirm that the siRNAs did knockdown the host factors, lysates used to examine HBV polymerase levels were probed to detect the five host factors. All five host factors were specifically knocked down by their cognate siRNA (Figure S6). The knockdown of GRP78 also caused a reduction in the levels of RPLP1 and RPLP2, consistent with the notion that these three host factors interact within the cell.

A modest reduction of total HBV RNA in cells treated with increasing concentrations of siRNA targeting siHNRNPA2B1. SiRNA knockdown of SRSF1 and HNRNPA2B1 in HepG2.2.15 cells resulted in a ca. 50% reduction of the total HBV RNA (Figure S3E). This was examined further by transfecting cells with multiple concentrations of siHNRNPA2B1 and quantifying the total HBV RNA using a Quantigene assay. In the same experiment, cells transfected with ALG-10000 yielded comparable results (Figure S7). Finally, ALG-20002, an antisense oligonucleotide that directs the cleavage of the HBV RNA, resulted in a large reduction of the total HBV RNA.

Characterization of the STOPS-binding host factors in HBV-infected primary human hepatocytes. We sought to validate that the host factors have roles in HBV infection in primary

human hepatocytes (PHHs). HBV-infected PHHs were transfected with 20 nM of each of the five siRNAs. The siRNAs targeting GRP78, RPLP1, RPLP2, and SRSF1 resulted in reduced HBsAg levels (Figure S8A). The knockdown of HNRNPA2B1 did not affect HBV HBsAg production in the HBV-infected PHHs (Figure S8B). Western blots of the PHHs lysates revealed that HNRNPA2B1 was knocked down. These results are reproducible with PHHs from two independent donors. HNRNPA2B1 is, therefore, less important for HBsAg production in PHHs than in HepG2.2.15 cells.

We examined the dose responses of the siRNA in knocking down GRP78, RPLP1, RPLP2, and SRSF1 to reduce HBsAg in PHHs. All four siRNAs have EC_{50} values of less than 1 nM, somewhat lower than the concentration needed to reduce HBsAg in HepG2.2.15 cells. The top-level of inhibition, 60-67%, was lower than that observed in HepG2.2.15 cells (ca. 75-90%). These differences may be due to the longer time in which the siRNAs were transfected into the PHHs (six days) compared to the HepG2.2.15 cells (three days). Notably, the siRNAs did not exhibit observable cytotoxicity in PHHs.

ALG-10000 and the knockdown of the five host factors that bind ALG-10000 only modestly reduced the level of total HBV RNA level in HepG2.2.15 cells. We wanted to determine whether there are comparable effects in HBV-infected PHHs. PHHs transfected with 20 nM ALG-10000 and one or more siRNAs (10 nM each) to knock down the host factors were infected with HBV as per the protocol in Figure 4D. After six days, the cells were lysed and the RNAs were quantified using a Quantigen assay. HBV RNA was only decreased by ca. 50% by ALG-10000 and all five siRNAs (Figure S8C). The results in HBV-infected PHHs mirror those in HepG2.2.15 cells (Figure S8D).

ALG-10000 increases polyubiquitination of the HBsAg in HepG2.2.15 cells. In addition to the reduction in HBsAg production, the decrease in HBsAg abundance with ALG-10000 may be due to an increase in the rate of HBsAg degradation rate. Misfolded proteins are typically ubiquitinated and then degraded by the proteasome.[7] To examine ubiquitination, lysates from HepG2.2.15 cells were immunoprecipitated for the HBsAg, adjusted to the same protein concentration, separated on denaturing PAGE then probed with an antibody conjugated to the enzyme horseradish peroxidase in a Western blot. The procedure used the buffers and components from a vendor (catalog # BK161, Cytoskeleton Inc.). HBsAg migrated as a smear expected of highly polyubiquitinated proteins, even in mock-treated cells. However, in cells treated with ALG-10000 for 76 h, polyubiquitinated HBsAg increased ca. 1.5-fold relative to that of mock-treated cells (Figure S9). In cells treated with ALG-10000 for 88 h, the relative amount of ubiquitinated HBsAg increased to 2.6-fold. These results show that a proportion of the HBsAg made in HepG2.2.15 cells may be normally targeted for degradation and that ALG-10000 increased the amounts of the proteins to be degraded.

HBV RNA sequences are required for the function of GRP78, RPLP1 and RPLP2 on HBsAg production. We examined whether RPLP1 and RPLP2 and GRP78 will affect HBsAg produced from plasmids. The Small HBsAg expressed from plasmid pcDNA3.1 in HEK 293 cells was not negatively affected by either STOPS or the knockdown of all 5 host factors (Figure S8A). We also examined whether STOPS and the knockdown of the 5 host factors affect the expression of the Large S-antigen from plasmid pcDNA3.1. Western blots showed that STOPS and the knockdown of all five host factors did not affect the amounts of the Large S-antigen (Figure S10A). Western blots analysis showed that the host factors were indeed knocked down in the cells, ruling out the

trivial explanation that the siRNAs had failed. The Western blots also revealed that the Large S antigen migrated as compact bands, unlike the more post-translationally modified ones in HepG2.2.15 cells. These results suggest that post-translational modifications of the Large S-antigen expressed from a recombinant plasmid in HEK cells may differ than the forms expressed from the HBV genome in HepG2.2.15 cells.

We hypothesize that one or more of the host factors require additional RNA sequences in the HBV genome to function for HBsAg production. To examine this possibility, HBsAg was produced from plasmid p1.05Gen_PsiCHECK2 containing 105% of the HBV genome expressed from the SV40 promoter and polyadenylation site. HEK293 cells transfected with p1.05Gen_PsiCHECK2 produced significant amounts of extracellular HBsAg. In addition, knockdowns of GRP78, RPLP1, and RPLP2 significantly reduced the levels of the extracellular HBsAg. Knockdowns of SRSF1 did not affect the amounts of the HBsAg. As a positive control in this experiment, ALG-20002, an anti-sense oligonucleotide that targets a sequence 3' of the HBsAg dramatically reduced the extracellular HBsAg. These results show that when HBsAg was expressed with additional HBV sequences, reduced amounts of the GRP78, RPLP1, and RPLP2 prevent optimal HBsAg production.

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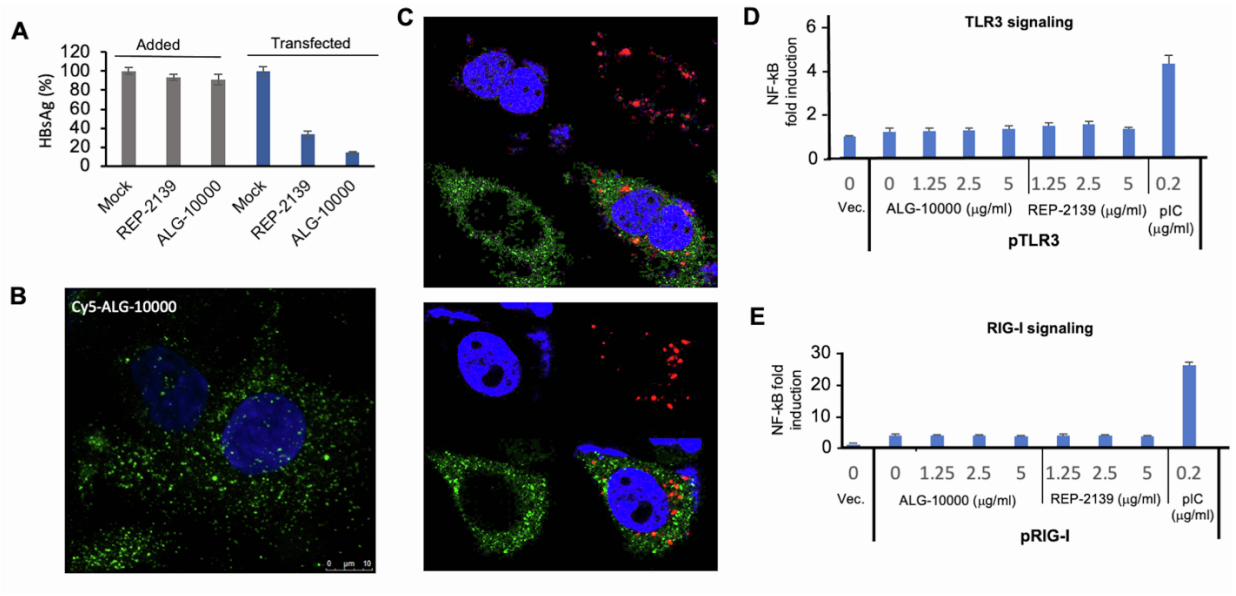

Figure S1. Basic properties of STOPS ALG-10000. A) ALG-10000 inhibits HBsAg only when transfected into HepG2.2.15 cells. ALG-10000 was added to the cell culture media at 100 nM and transfected into cells at 10 nM. REP 2139 was added at 200 nM and transfected into cells at 40 nM. **B**) ALG-10000 localizes mostly to the cytoplasm of HepG2.2.15 cells in discrete structures. Cy5-ALG-10000 was transfected at 10 nM into HepG2.2.15 cells and is pseudo-colored green in the micrograph. **C**) ALG-10000 does not extensively co-localize with HBsAg in transfected cells. HBsAg was pseudocolored green in the micrograph, and Cy5-ALG-10000 was pseudo-colored red. **D**) ALG-10000 does not activate signaling by the innate immune receptor TLR3. **E**) ALG-10000 does not activate signaling by the RIG-I innate immune receptor.

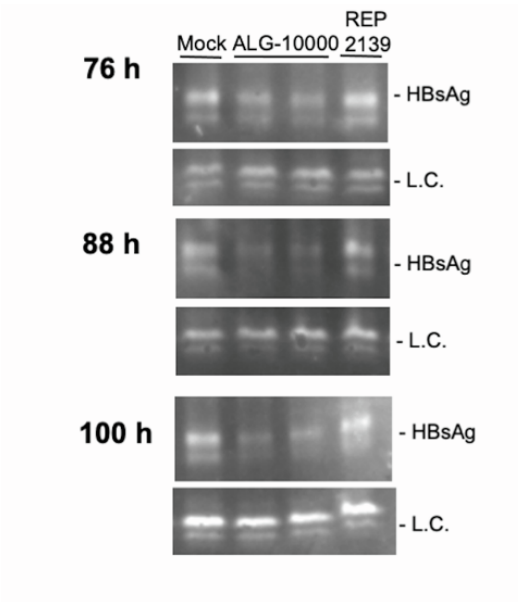

Figure S2. ALG-10000 can reduce the amount of intracellular HBsAg. The Western blot images contain the Large HBsAg present in HepG2.2.15 cell lysates collected at 76, 88 and 100 h after transfection with 8 nM ALG-10000 or 20 nM REP 2139. The loading control (L.C.) is a cellular protein that is recognized by the antibody.

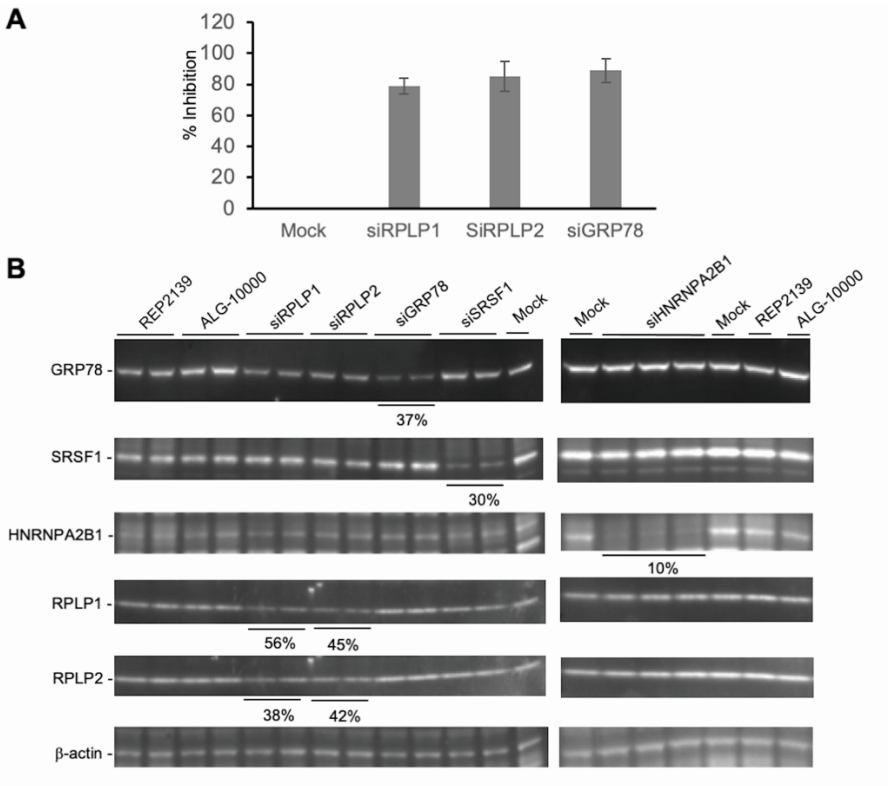

Figure S3. siRNAs knock down their specific host factors. **A**) Quantification of the decrease in host factor RNA after treatment with siRNAs. The RNAs were quantified from HepG2.2.15 cells mock-transfected or transfected for 72 h with 10 nM of siRNA targeting RPLP1, RPLP2 and GRP78 by real-time RT-PCR. **B**) Western blots demonstrating the knockdown of the host proteins by siRNAs targeting the host factors. The HepG2.2.15 cells were transfected with 10 nM of the five host factor siRNAs for 72 h. RPLP1 and RPLP2 were probed together in the same blot, as the two proteins form a heterodimer and each protein can stabilize the other.

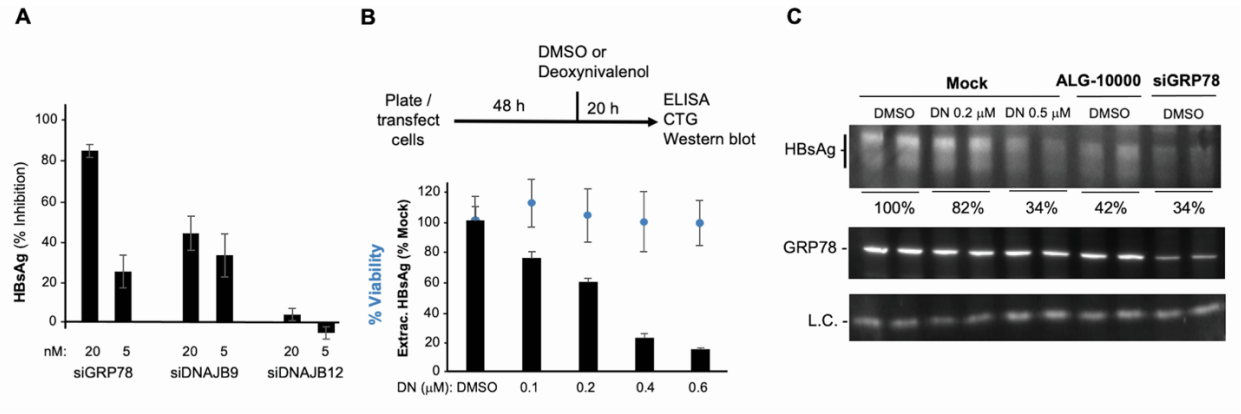

Figure S4. Western blots demonstrating that ALG-10000 can decrease SRSF1 levels. The loading control (L.C.) is the cellular protein GAPDH. The amount of protein reduction was normalized to that of the mock-transfected sample.

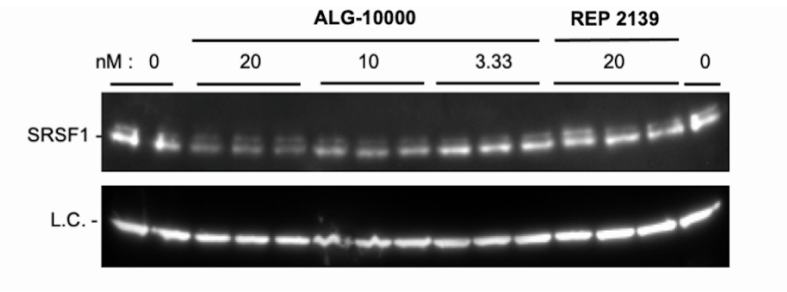

Figure S5. Validation of GRP78 functioning in HBV replication and the reduction of HBsAg. **A**) SiRNA knockdown of co-chaperone DNAJB9 that interacts with GRP78 can also reduce the amount of extracellular HBsAg. **B**) Deoxynivalenol (DN) a known inhibitor of GRP78, can reduce extracellular HBsAg produced by HepG2.2.15 cells. The schematic represents the protocol used. DN concentrations were selected to not affect the viability of HepG2.2.15 cells. **C**) DN reduces the amounts of intracellular HBsAg in a concentration-dependent manner. The top image of the Western blot shows the Large HBsAg. The middle image is of GRP78, whose abundance is reduced by siGRP78. The loading control (L.C.) is β-actin.

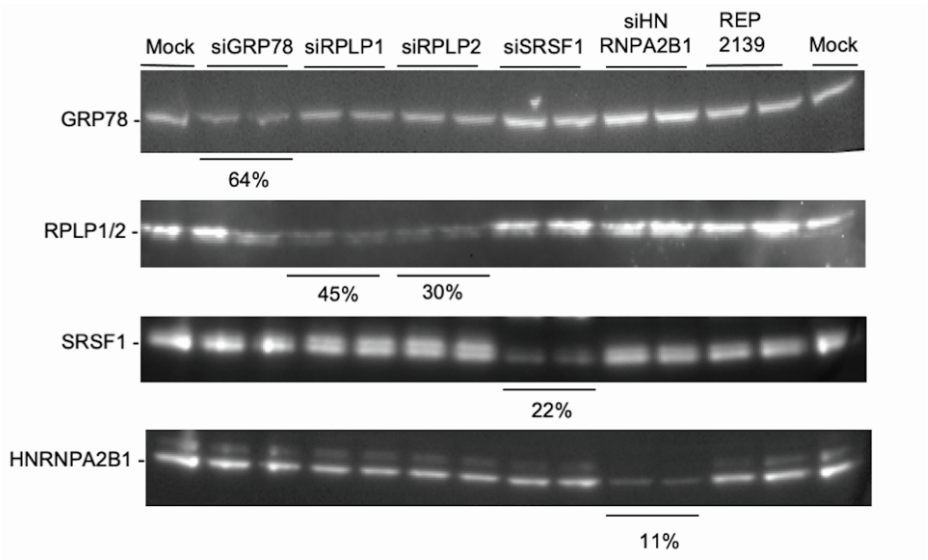

Figure S6. Confirmation that the siRNAs reduced their specific target proteins in the samples analyzed for the HBV polymerase.

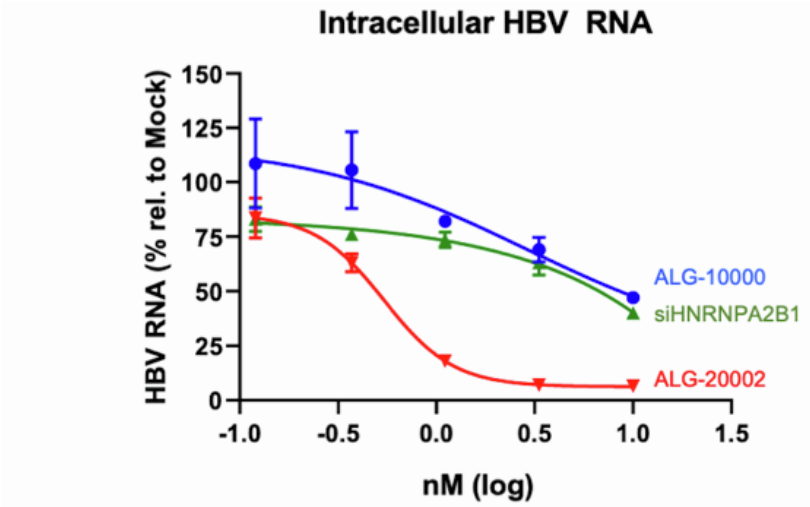

Figure S7. Effects of transfected siHNRNPA2B1, ALG-10000 and ALG-20002 on the total amount of the HBV RNA in HepG2.2.15 cells.

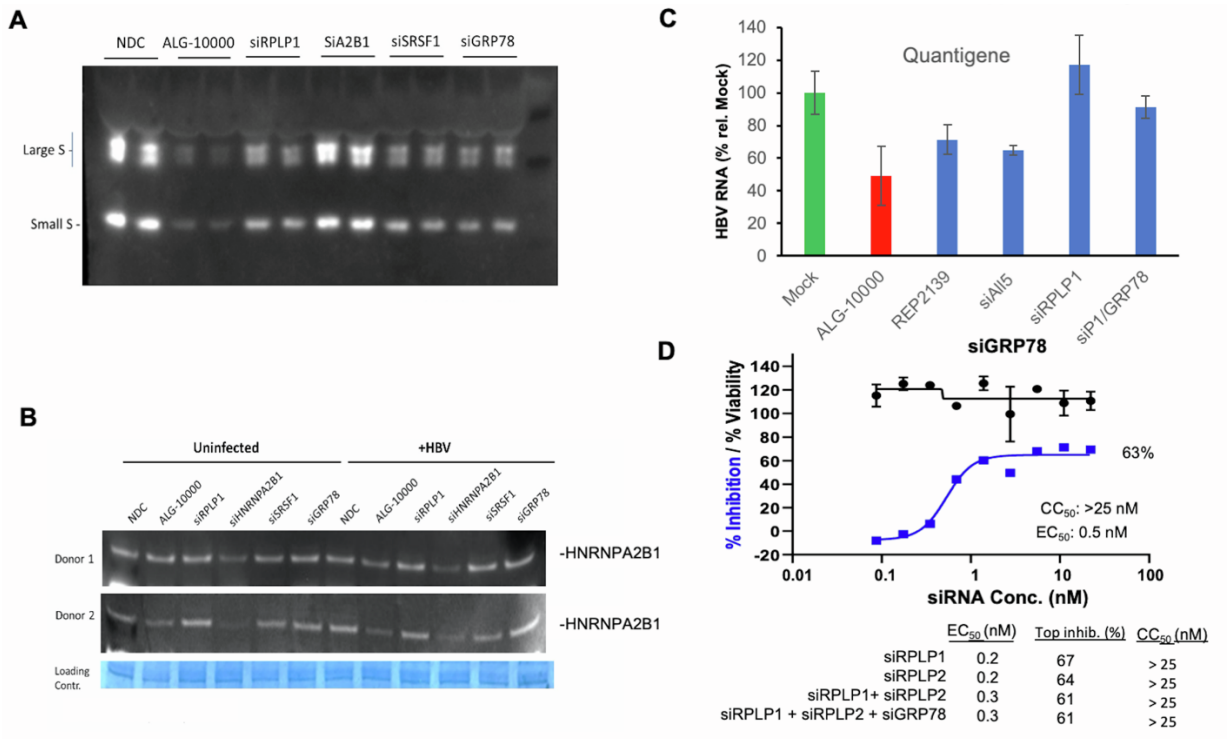

Figure S8. Characterizations of the host factor siRNAs function in PHHs. **A**) Western blot analysis of the HBsAg produced by HBV-infected PHHs. **B**) Western blot analysis of the HNRNPA2B1 protein in PHHs treated with ALG-10000 or siRNAs targeting host factors. **C**) Effects of the host factor siRNAs on total HBV RNAs. **D**) Dose responses of the host factor siRNAs on the reduction of extracellular HBsAg. A sample dose response curve for the inhibition of extracellular HBsAg levels and cell viability. The summary of the EC_{50} values for the siRNAs in PHH is located below the graph.

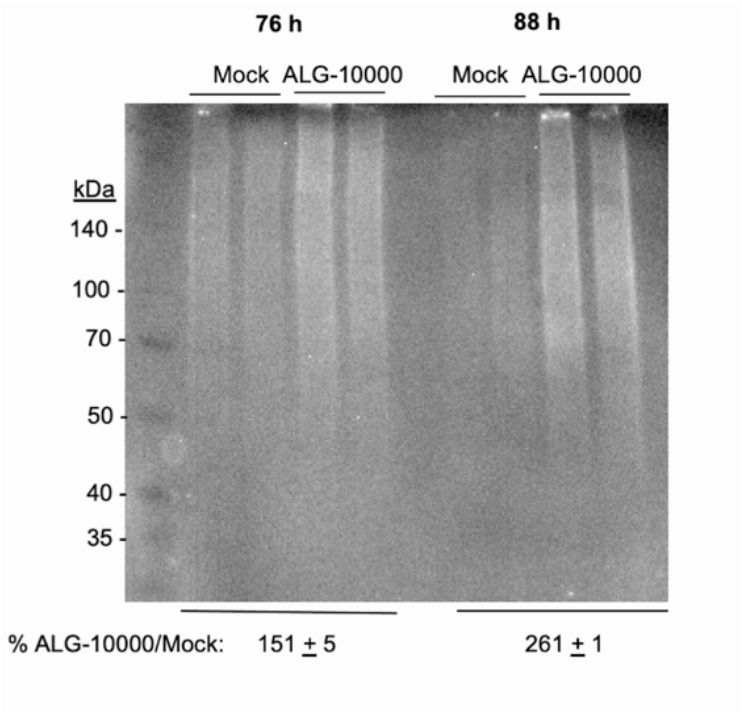

Figure S9. ALG-10000 can increase the polyubiquitination of the HBsAg. The Western blot image contains cell lysates of HepG2.2.15 cells mock-transfected or transfected with 10 nM ALG-10000 for 76 and 88 h.

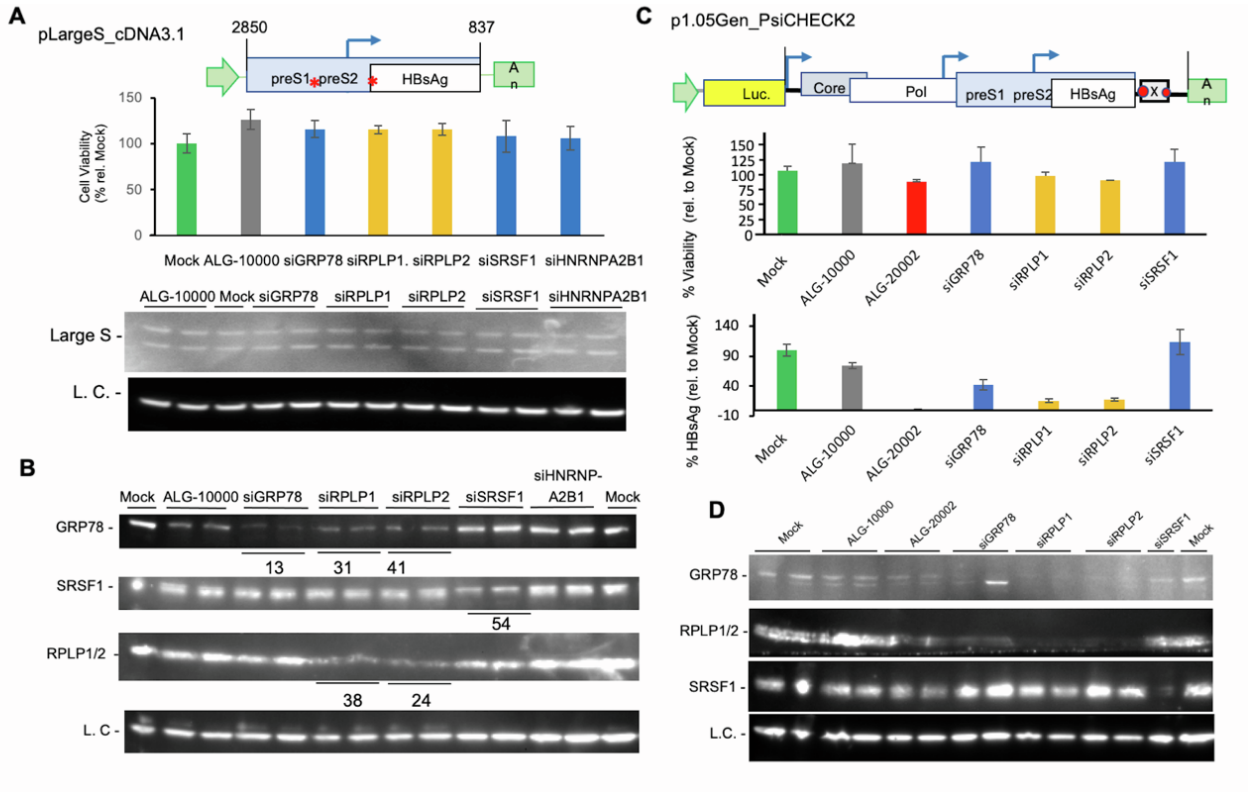

Figure S10. Host factors can affect HBsAg production when the HBsAg was expressed with regulatory RNA sequences. **A**) Expression of the Large S antigen from a plasmid construct lacking HBV regulatory sequences does not require the host factors. The schematic is of the Large HBsAg cloned into vector pCDNA3.1. The red asterisks denote that the translational initiation codon was mutated to prevent production of the Middle and Small S-antigens. The bar graph shows that the expression of the Large S antigen and transfection with the host factor siRNAs did not reduce the viability of the HEK293 cells. The loading control (L.C.) is GAPDH. **B**) Western blot images showing that the host factors were reduced in cells transfected with siRNAs. **C**) Expression of the HBsAg from a construct containing the entire HBV genome. The schematic shows the HBV sequence cloned in plasmid p1.05Gen_psiCHECK2. The upper bar graph shows the effect of the manipulations on cell viability, as assessed using the CellTiterGlo reagent. The lower bar graph shows the amount of extracellular HBsAg produced from p1.05Gen_psiCHECK2 by HEK293

cells 54 h after the cells were transfected with oligonucleotides and 36 h after transfection with p1.05Gen_psiCHECK2. All oligonucleotides were transfected at 10 nM and p1.05GEN_psiCHECK2 was transfected at 100 ng/well. **D**) Western blot images show the amounts of the Large S antigen and the loading control (L.C.), GAPDH, present in the cell lysate.