Supplementary Material

Therapeutic shutdown of hepatitis B virus transcripts promotes reappearance of the SMC5/6 complex and silencing of the viral genome in vivo

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Supplementary methods

Generation of USG mice. All animal experiments were performed in accordance with the European Union directive 2010/63/EU and approved by the ethical committee of the city and state of Hamburg in accordance with the ARRIVE guidelines. Animals were maintained under specific pathogen-free conditions in accordance with institutional guidelines under approved protocols. One million thawed human hepatocytes were injected intrasplenically into 3-week-old homozygous mice anesthetized with isoflurane. After eight weeks, the levels of human chimerism were determined by measuring human serum albumin in mouse serum with the Human Albumin ELISA kit (Immunology Consultants Lab, Portland, USA). HBV infection was established upon a single intraperitoneal injection of HBV-containing mouse serum ($1x10^7$ HBV DNA copies/mouse, genotype D, HBeAg-positive). Blood samples were taken retro-orbitally

during the experiments. Liver specimens removed at the time of sacrifice were snap-frozen in 2-methylbutane and stored at -80°C for histological and molecular analyses.

Virological measurements. Viral DNA was extracted from serum samples using the QiAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). QPCR measurements were performed on the ViiA 7 Real-Time PCR System with probes and primers from the TaqMan® Gene Expression Assay System and the Taqman® Fast Advanced Master Mix (Thermo Fisher, Waltham, Massachusetts, USA)(1). Viral titers in the serum were quantified with HBV-specific primers and probes (TaqMan® Gene Expression assay ID: Pa03453406_s1) while known references of an HBV DNA plasmid were amplified to establish a standard curve for absolute quantification. HBsAg and HBeAg quantification were performed on the Abbott Alinity I platforms (quantitative HBsAg kit, and HBeAg kit, Abbott, Ireland, Diagnostic Division) after diluting the mouse serum 1:200 in the dilution serum (Abbott) as recommended by manufacturer. DNA and RNA were extracted from liver specimens using the Master Pure DNA purification kit (Epicentre, Madison, USA) and the RNeasy Mini Kit (Qiagen), respectively. Intrahepatic HBV DNA was quantified with the same primer and probe set used for serum. For normalization, the number of human hepatocytes was estimated by measuring human hemoglobin beta (assay ID Hs00758889_s1) while human genomic DNA (Roche Applied Science, Mannheim, Germany) was used in a standard curve for quantification. Intrahepatic HBV RNA levels were determined in a one-step qRT-PCR using the TaqMan® Fast Virus 1-Step Master Mix as recommended by the manufacturer (Thermo Fisher). The same HBV-specific primer and probe set used for serum amplified intrahepatic total HBV RNA. Pregenomic HBV RNA (including precore HBV RNA) was amplified as described elsewhere(2). The expression of two human housekeeping genes (GAPDH; glyceraldehyde-3-phosphate dehydrogenase, assay ID Hs99999905_m1, and RPL30; ribosomal protein L30, Hs00265497_m1) was used for normalization. All intrahepatic measurements were performed on three different liver pieces isolated per mouse.

cccDNA quantification. For Southern blot analysis, DNA was extracted with a modified protocol using the Master Pure DNA purification kit without the proteinase K digestion step. Omitting the protein digestion results in reduced precipitation of protein-bound DNA and subsequently lower levels of rcDNA compared to the classical extraction with proteinase K. To further reduce non-cccDNA HBV DNA forms, which might give rise to background staining on the blot, all samples were digested with plasmid-safe ATP-dependent DNase (Epicentre) using the following conditions: 1 µg total DNA was incubated with 30 U of enzyme at 37°C in a total volume of 200 µl for 2 h. After heat inactivation (30 min at 70°C), the DNA was purified using the DNA clean and concentrator kit (Zymo Research, Irvine, California, USA). Before loading,

a small part of every sample was set aside, diluted and used for cccDNA PCR for a direct comparison of qPCR and Southern blot quantification. qPCR with cccDNA-selective TaqMan primers and probe(2) was performed using modified PCR conditions (final concentration of forward primer 100 nmol/l; reverse primer 800 nmol/l; cycling conditions: 10 min initial denaturation at 95°C followed by 40 cycles of 95°C for 1 sec and 65°C for 1 min). Copy numbers of cccDNA molecules were normalized to human mitochondrial DNA present in the undigested column-purified samples by using human-specific primers and probe for the mitochondrial gene ND2 (assay ID #Hs02596874_g1). Southern blot analysis was performed as previously described(3) with minor modifications. Because liver samples from chimeric mice harbor varying amounts of human hepatocytes, samples were normalized to the amount of human mitochondrial DNA measured by qPCR in undigested samples (see above) making sure that equal amounts of DNA originating from human hepatocytes were loaded in every lane. Samples were mixed with loading dye (New England Biolabs, Ipswich, Massachusetts, USA) and loaded onto a 1.2% TAE agarose gel together with a 1 Kb DNA ladder (Thermo Fisher). After gel electrophoresis at 25 V for 21 h, the gel was treated as follows: 10 min in depurination buffer (0.2N HCl), 1 h in denaturing buffer (0.5N NaOH/1.5M NaCl), 1 h in neutralization buffer (1.5M NaCl/1M Tris-HCl pH 7.4), 30 min in 20xSSC buffer (3M NaCl/0.3M sodium citrate) gently shaking at room temperature. The DNA was blotted onto a Nytran SPC membrane using the TurboBlotter kit (GE Healthcare, Chicago, Illinois, United States). After transfer, the membrane was rinsed in 2xSSC and UV-crosslinked at 254 nm and 120 mJ/cm^2. HBV DNA was detected with the help of branched DNA technology using the QuantiGene Singleplex assay (Thermo Fisher). Briefly, the membrane was blocked in hybridization buffer (30 min at 55°C), then hybridized with HBV DNA probes (assay ID #VF1-12525) overnight at 55°C. The following day, the membrane was successively incubated with the pre-amplifier (30 min at 55°C), amplifier (1 h at 55°C) and label probe (1 h at 50°C) before the signal was detected with CDP-STAR Detection Reagent (Roche, Basel, Switzerland) in a Fusion FX (Vilber Lourmat, Eberhardzell, Germany). Densitometric analysis was used to quantify the signal intensity of the cccDNA band (2.1kb) after rolling ball background subtraction in the Fusion FX software.

Gene expression analysis. Expression levels of human genes were quantified by qPCR as previously described(4). Briefly, liver RNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using oligo-dT primer and subjected to qPCR using the following human-specific primers not cross-reacting with murine sequences: SMC5 (TaqMan® Gene Expression assay ID #Hs00390892_m1), SMC6 (#Hs01093459_m1), ISG15 (#Hs00192713_m1), OAS1 (#Hs00973637_m1), HLA-A (#Hs01058806_g1), STAT1 (#Hs01013989_m1). Expression levels were normalized to the mean of two human

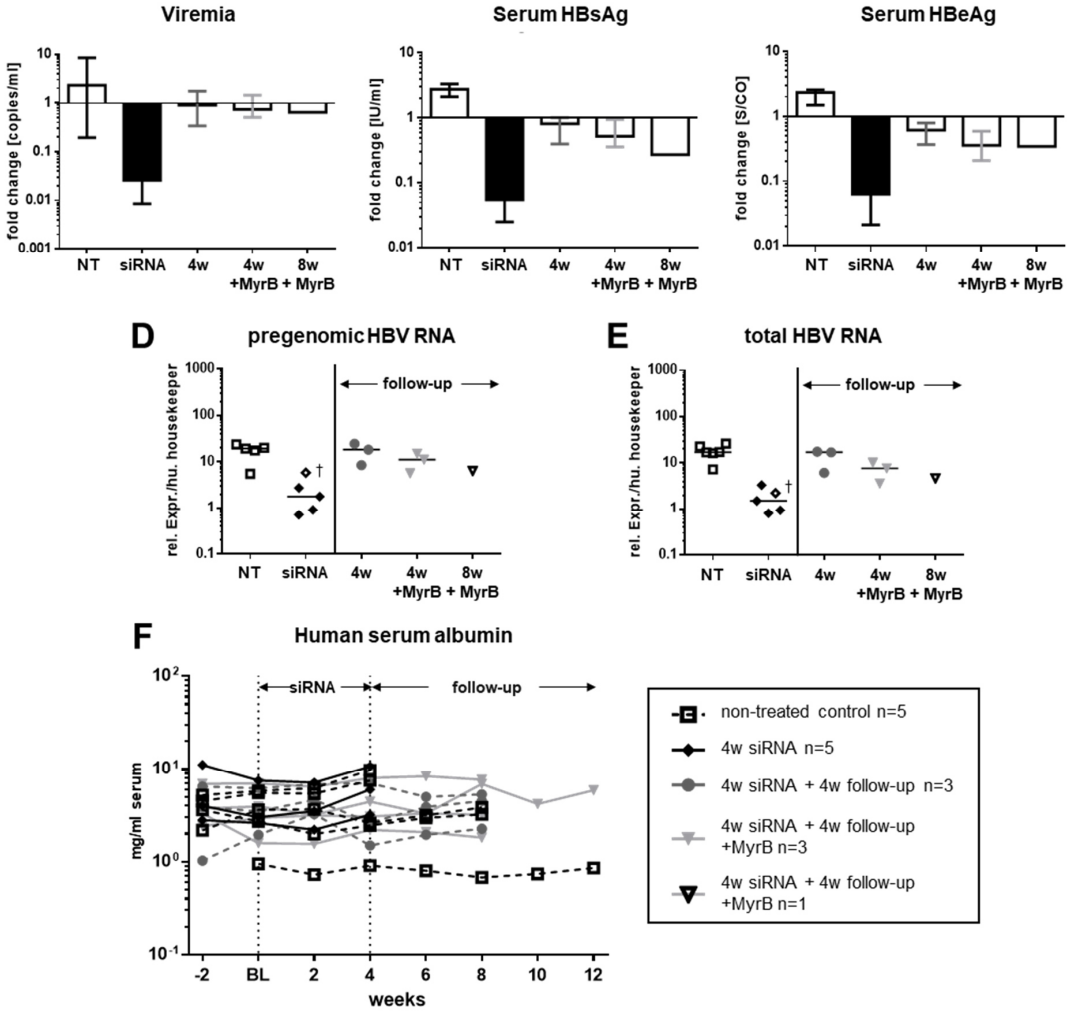
housekeeping genes (GAPDH and RPL30) as described above. Three different human hepatocyte donors were used for the engraftment of liver chimeric mice in this study. Since basal gene expression levels may vary from donor to donor for some genes, all treated mice were normalized to the expression levels of untreated control mice repopulated with the respective hepatocyte donor.

Chromatin Immunoprecipitation (ChIP). ChIP qPCR assays were performed as described previously(5) and adapted to the use of liver tissue. Briefly, liver tissues were pulverized in liquid nitrogen and resuspended in PBS containing proteases inhibitors with subsequent homogenization by douncing with loose pestles. Samples were fixed under constant rotation in methanol-free 1% formaldehyde for 10 min and quenched with 125 mM glycine for 5 min. Nuclei were isolated before chromatin extraction and chromatin was fragmented by sonication (Bioruptor Plus, Diagenode, Seraing Belgium), aliquoted and kept frozen at -80 °C until further use. Chromatin was precipitated with 3 µl anti-NSE4 (#AP9907-a, Abgent, San Diego, USA) or 1 µl unspecific rabbit IgG using the MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher) according to the manufacturer´s instructions. cccDNA in the input and precipitates was amplified by qPCR using TaqMan primer and probe as described above.

Immunofluorescence. Immunofluorescence stainings were performed on 12-µm cryostat liver sections. Human SMC6 was co-stained with HBcAg on liver sections after fixation in 4% paraformaldehyde using a polyclonal rabbit anti-SMC6 antibody (#HPA042733, Sigma Aldrich), which does not cross-react with mouse Smc6, and a monoclonal mouse anti-HBcAg antibody (clone 10E11, # ab8639, Abcam, Cambridge, United Kingdom). Specific signals were visualized with Alexa Fluor 488 or 555 labeled secondary antibodies (Invitrogen, Darmstadt, Germany). Nuclear staining was achieved by Hoechst 33258 (Invitrogen, Eugene, USA). Stained sections were analyzed by fluorescence microscopy (BZ-X710, Keyence, Osaka, Japan) using the same settings for all mice per staining.

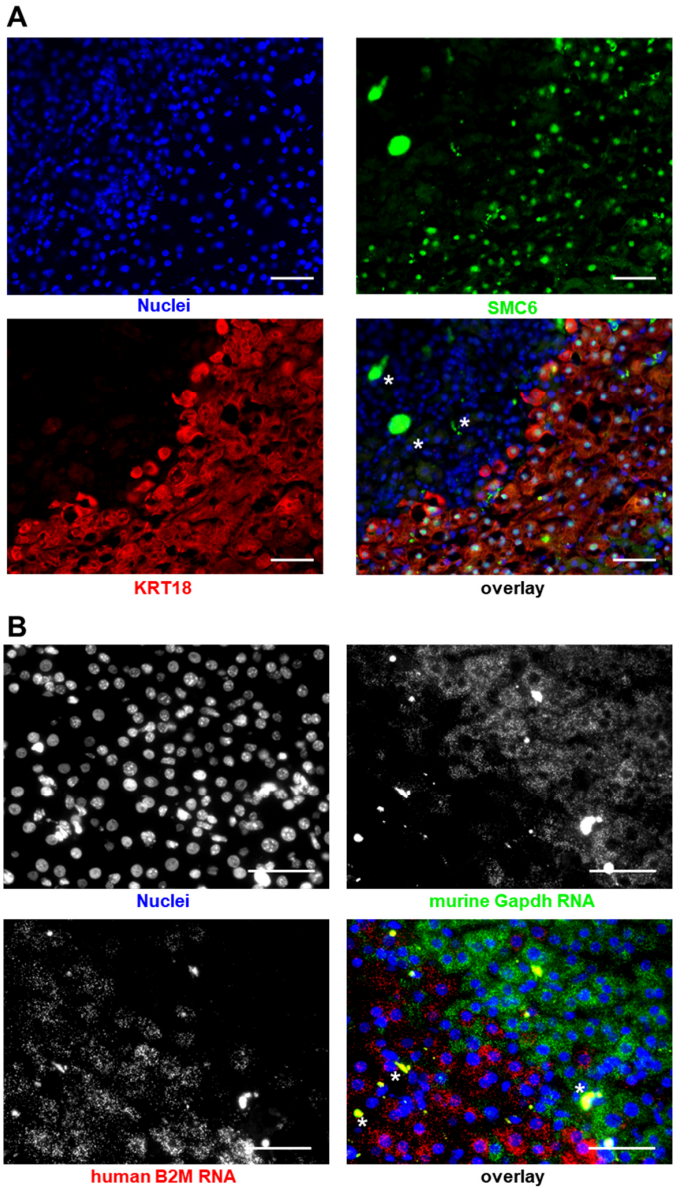
Statistical Analysis. Reductions from baseline were calculated from individual mice and compared between treated and untreated groups at the end of treatment using the Mann-Whitney test and GraphPad Prism 6. P-values <0.05 were considered statistically significant.

Supplementary figures



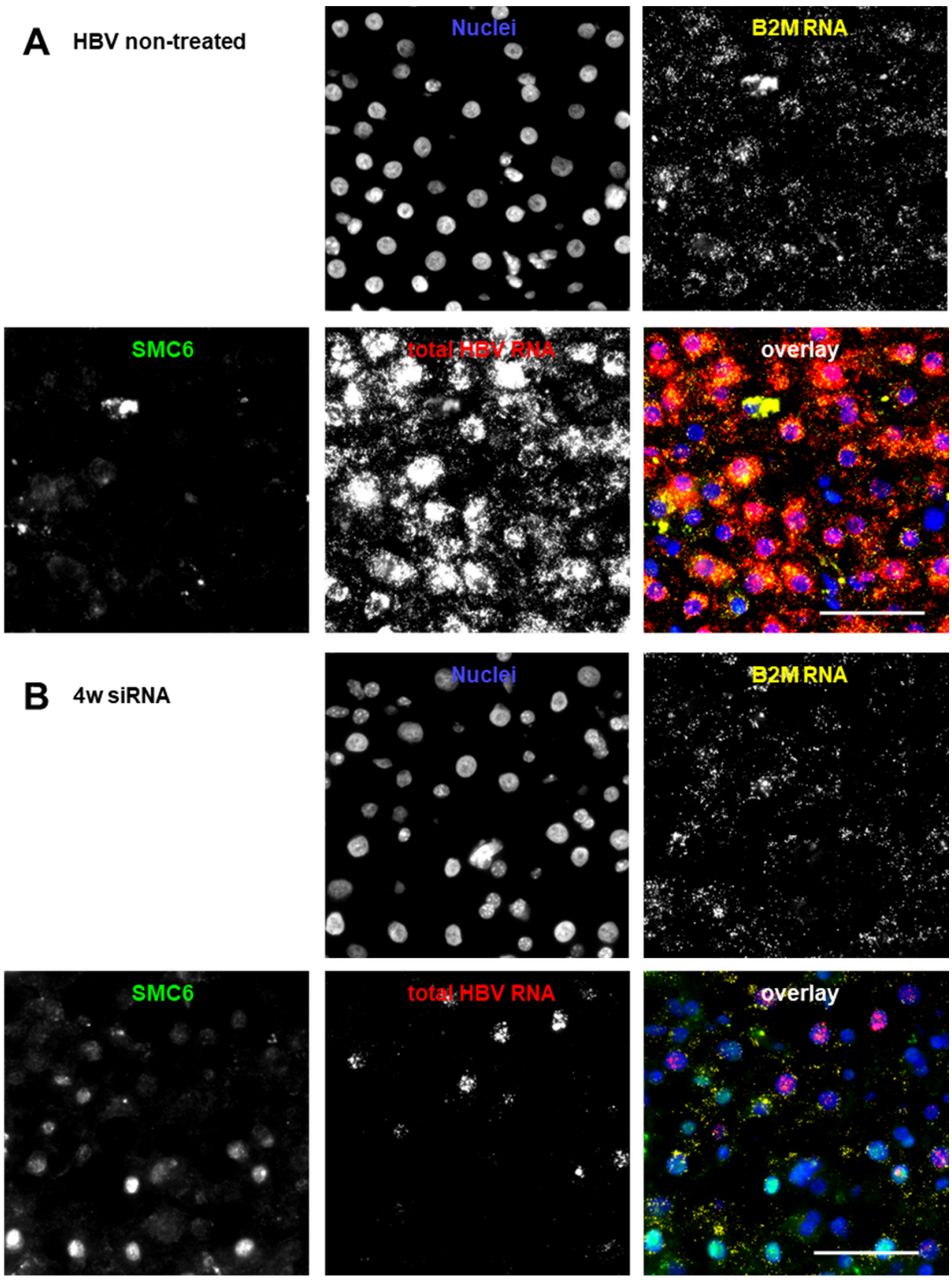
Suppl. Fig. 1: Viral DNA, RNA and protein levels are reduced but human serum albumin levels do not change upon siRNA treatment

Mice were sacrificed at the indicated time points and changes of serum HBV DNA levels (A), HBsAg (B) and HBeAg (C) were determined by qPCR or ELISA and depicted as bar graphs showing the median and range. Pregenomic HBV RNA (D) and total HBV RNA levels (E) normalized to human housekeeping genes were determined by qPCR. Each dot represents a single mouse; horizontal lines depict the median. (F) Longitudinal analysis of human serum albumin levels as determined by ELISA in mouse serum. Mice were treated as indicated below the graph and blood was drawn every other week. Every line represents one mouse. NT, non-treated.



Suppl. Fig. 2: SMC6 protein and B2M RNA staining are human-specific.

(A) Immunofluorescence co-staining for SMC6 (green) and cytokeratin 18 (KRT18) (red) in cryopreserved liver sections of an untreated non-infected USG mouse. KRT18 was used as a specific marker for human hepatocytes. Nuclei were stained with Hoechst 33258. (B) RNA-ISH co-staining for murine Gapdh transcripts (green) and human B2M transcripts (red) in cryopreserved liver sections of an untreated HBV-infected USG mouse. Nuclei were stained with DAPI. Scale bar 50 µm. * indicates autofluorescent phagocytic remnants.


A HBV non-treated
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay
B 4w siRNA
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay

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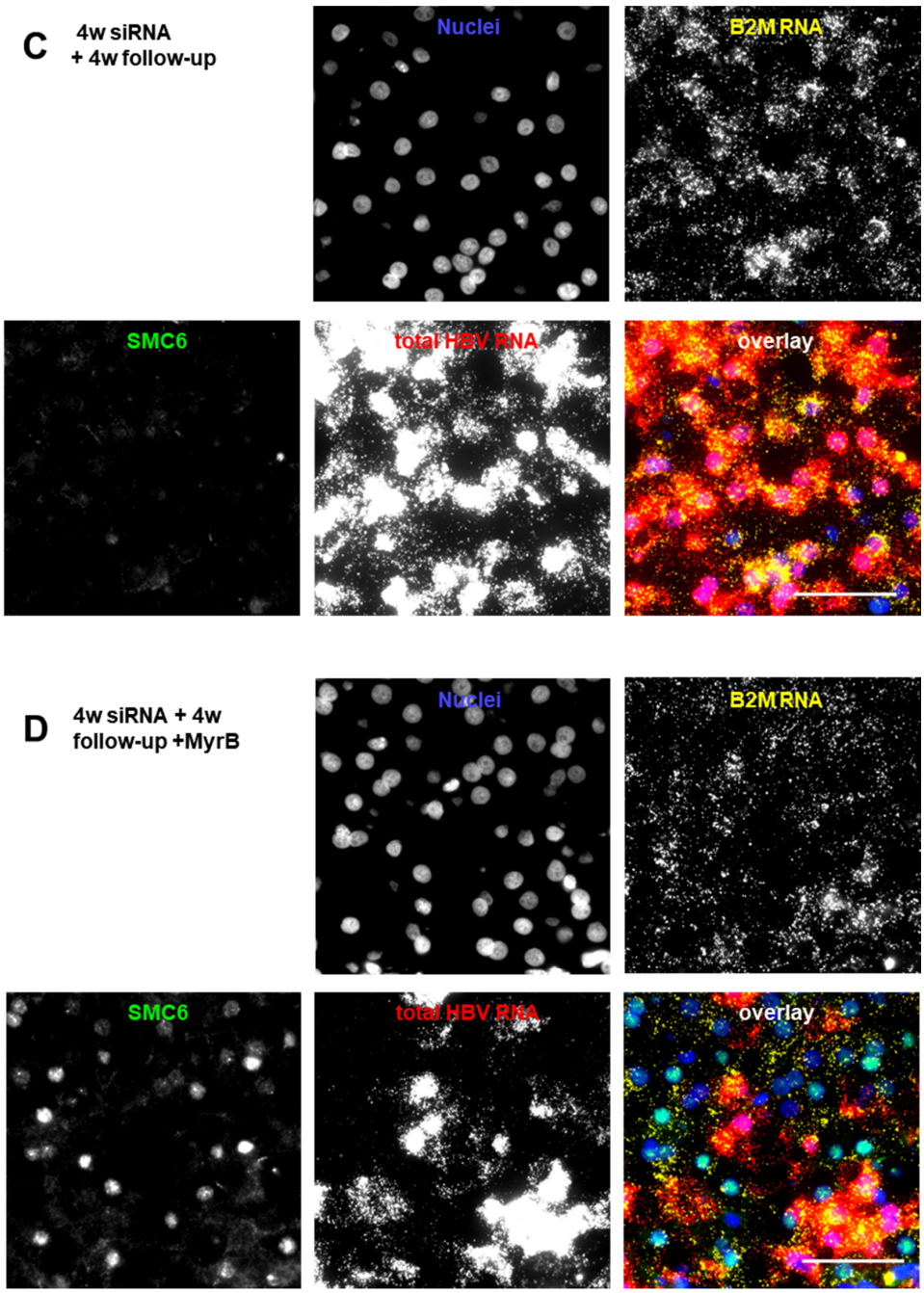
C
4w siRNA
+ 4w follow-up
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay
D
4w siRNA + 4w
follow-up +MyrB
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay

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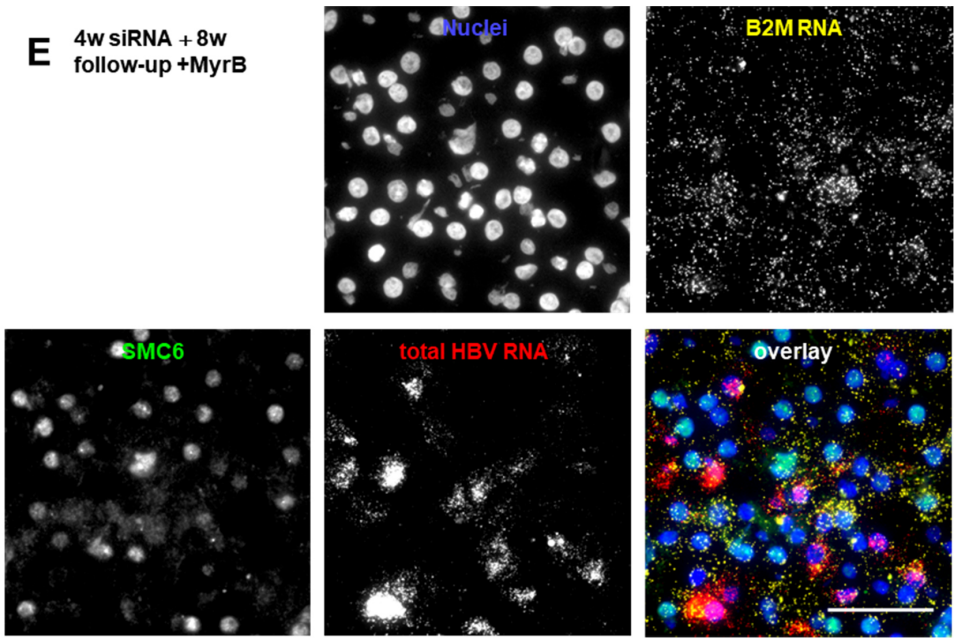
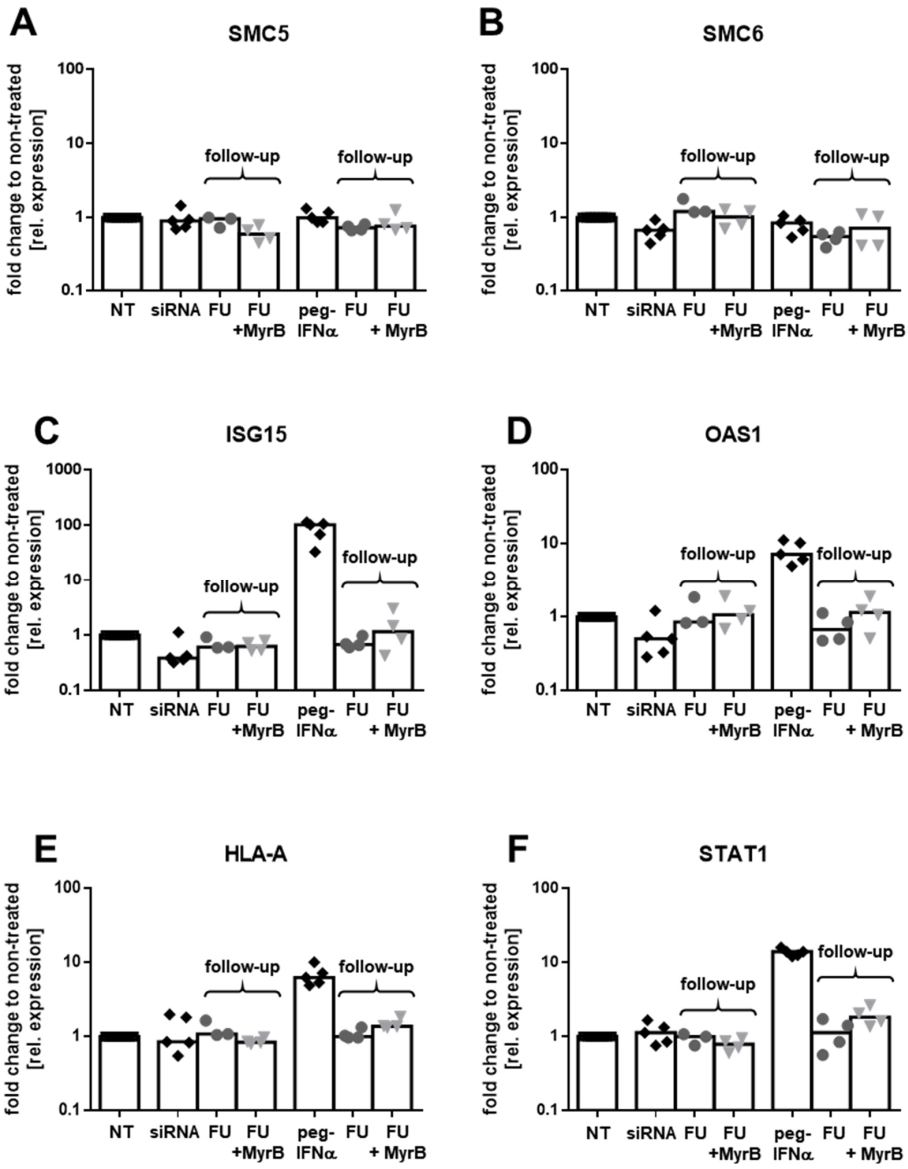


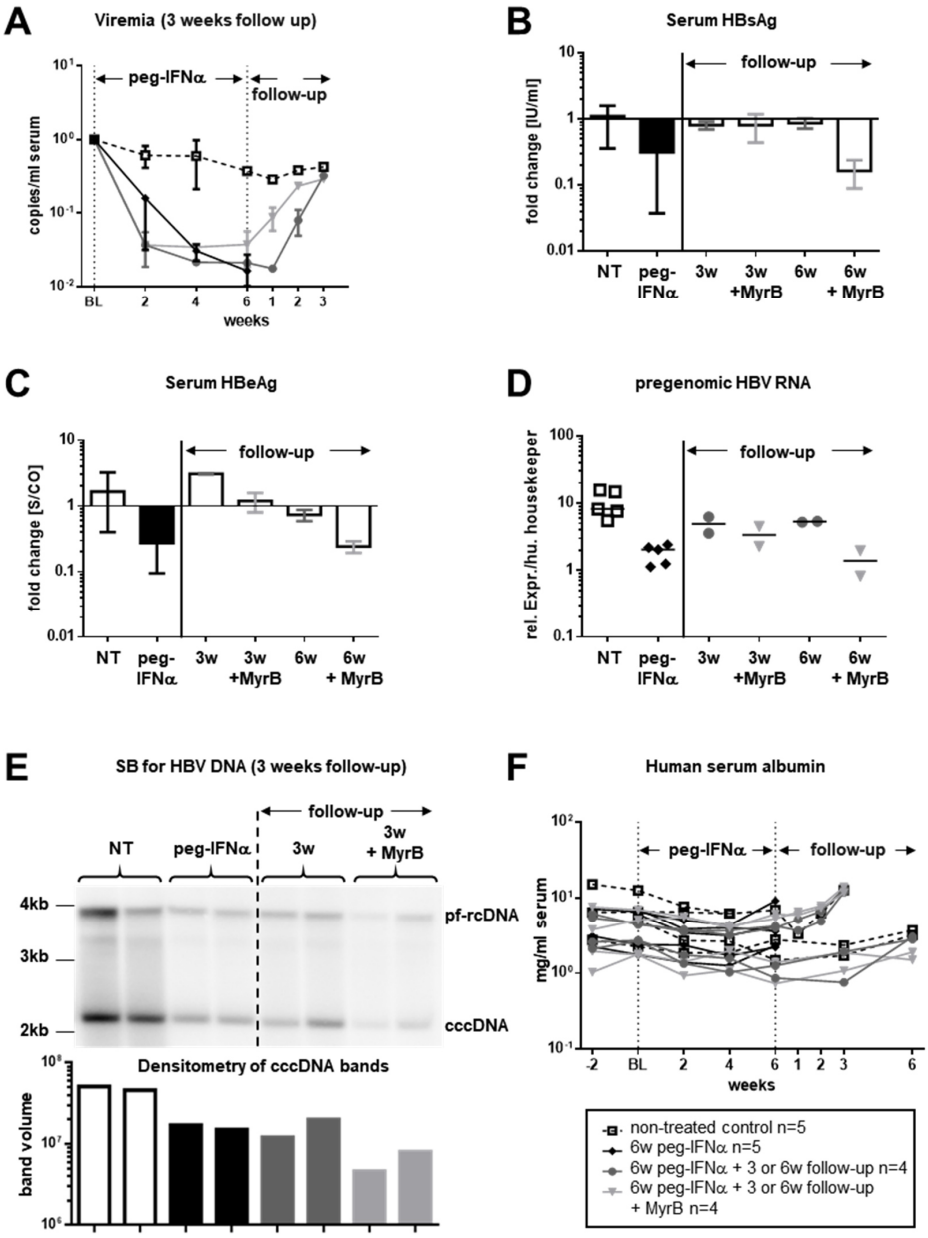
Suppl. Fig. 3: siRNA treatment leads to the reappearance of SMC6 in hepatocytes negative for signs of active HBV replication.

(A-E) Single channel photographs and overlays of RNA in situ hybridization for total HBV RNA combined with immunofluorescence staining for SMC6 protein. Shown are the same photographs as in figure 2 (representative pictures of one mouse from every treatment group as indicated at the left-hand side). Overlay pictures show nuclei stained with DAPI in blue, B2M RNA as a marker for human hepatocytes in aqua, SMC6 protein in green and total HBV RNA in red. Scale bar 50 µm.



Suppl. Fig. 4: Interferon-stimulated genes are induced upon peg-IFNα but not siRNA treatment while SMC5 and SMC6 mRNA levels remain stable across all treatments.

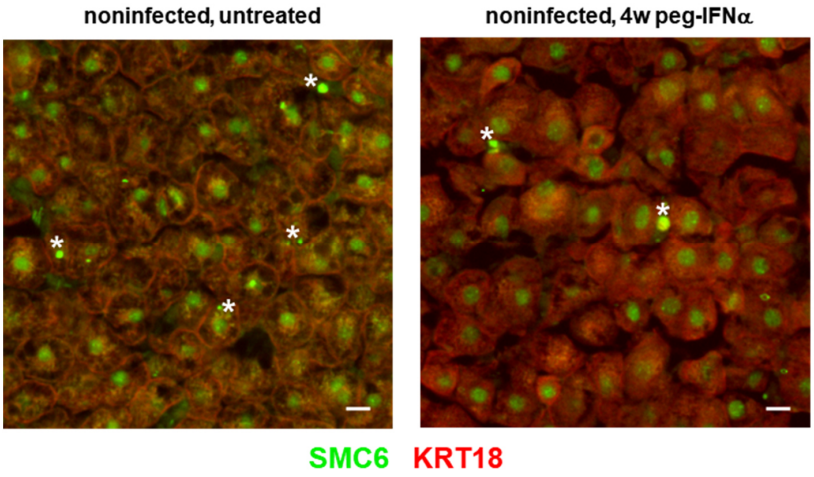
mRNA levels of SMC5 and SMC6 and classical human ISGs were determined by qPCR in all mice included in this manuscript using human-specific TaqMan primer and probe sets. The expression levels of SMC5 (A), SMC6 (B), ISG15 (C), OAS1 (D), HLA-A (E) and STAT1 (F) are shown as dot blots where bars depict the median and every dot represents a single mouse. Expression levels are normalized to the mean of two human housekeeping genes. These relative expression levels are then normalized to expression levels in untreated control mice repopulated with hepatocytes from the same human donor. NT, non-treated; FU, follow-up.



Suppl. Fig. 5: Viral markers including cccDNA are reduced upon peg-IFNα treatment without affecting human serum albumin levels

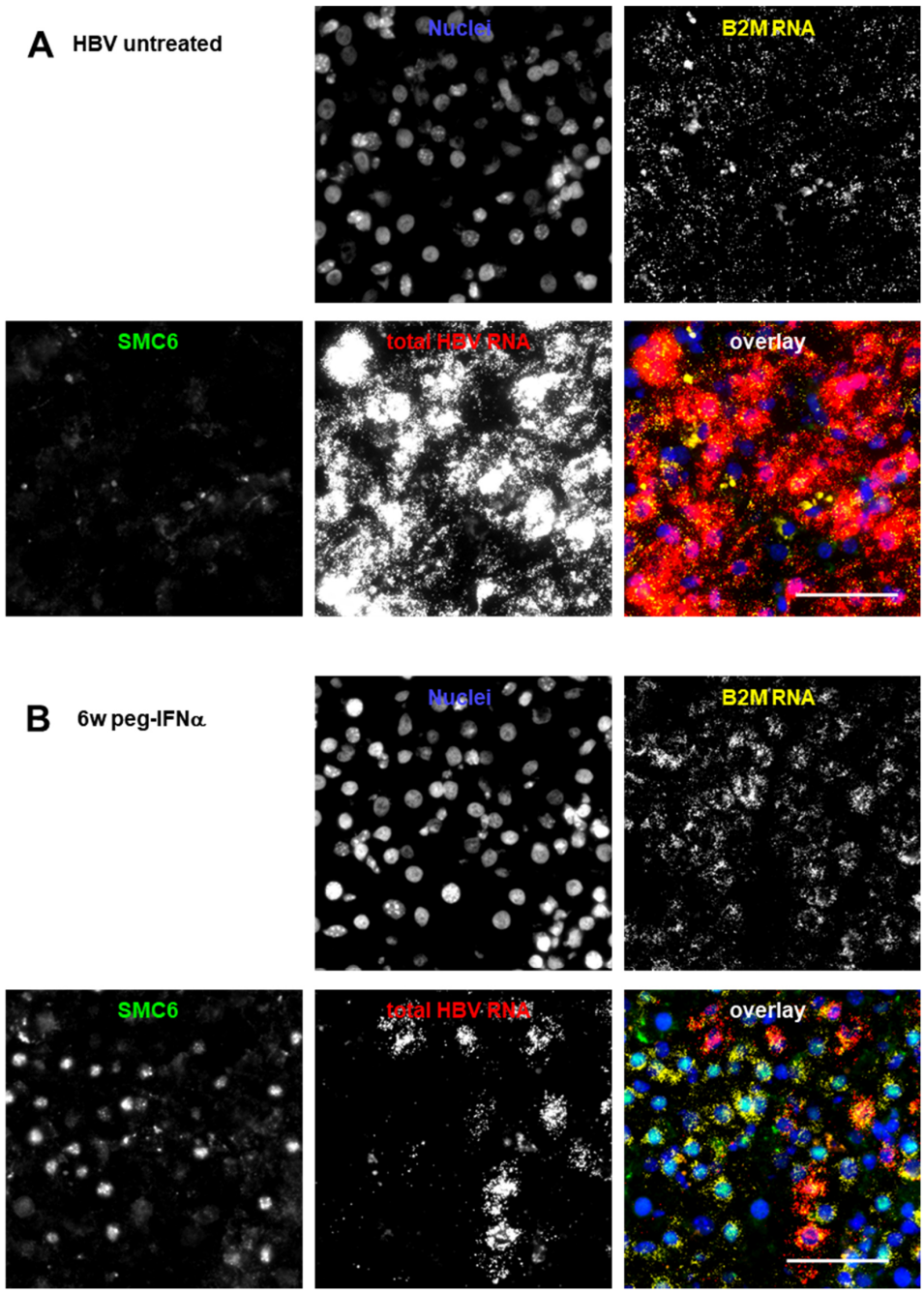
HBV-infected USG mice were treated with peg-IFNα as indicated in two independent experiments. (A) Blood was drawn every other week and viral titers were determined by qPCR. The line graph shows mice from experiment 1. Lines depict the median and error bars the range. (B, C) Changes of serum HBsAg (B) and HBeAg (C) from both experiments were determined by ELISA and depicted as bar graphs showing the median and range. (D) Mice were sacrificed at the indicated time points and pregenomic HBV RNA normalized to human

housekeeping genes was determined by qPCR. Each dot represents a single mouse; horizontal lines depict the median. (E) Liver DNA extracts (Epicentre-base extraction without proteinase K) were subjected to Southern blot. DNA amounts were normalized to human mitochondrial DNA and digested with PSD before loading. The bar graph below shows the densitometry analysis of the cccDNA band. The blot shows mice from experiment 1. pf-rcDNA, protein-free rcDNA; NT, not treated. (F) Longitudinal analysis of human serum albumin levels as determined by ELISA in mouse serum. Every line represents one mouse.



Suppl. Fig. 6: SMC6 protein levels in human hepatocytes are not affected by peg-IFNα treatment

Immunofluorescence co-staining for SMC6 (green) and cytokeratin 18 (KRT18) (red) in cryopreserved liver sections of an untreated non-infected USG mouse (A) and a non-infected USG mouse treated for four weeks with peg-IFNα (B). KRT18 was used as a specific marker for human hepatocytes. Scale bar 10 µm. * indicates autofluorescent phagocytic remnants.


A HBV untreated
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay
B 6w peg-IFNα
Nuclei
B2M RNA
SMC6
total HBV RNA
overlay

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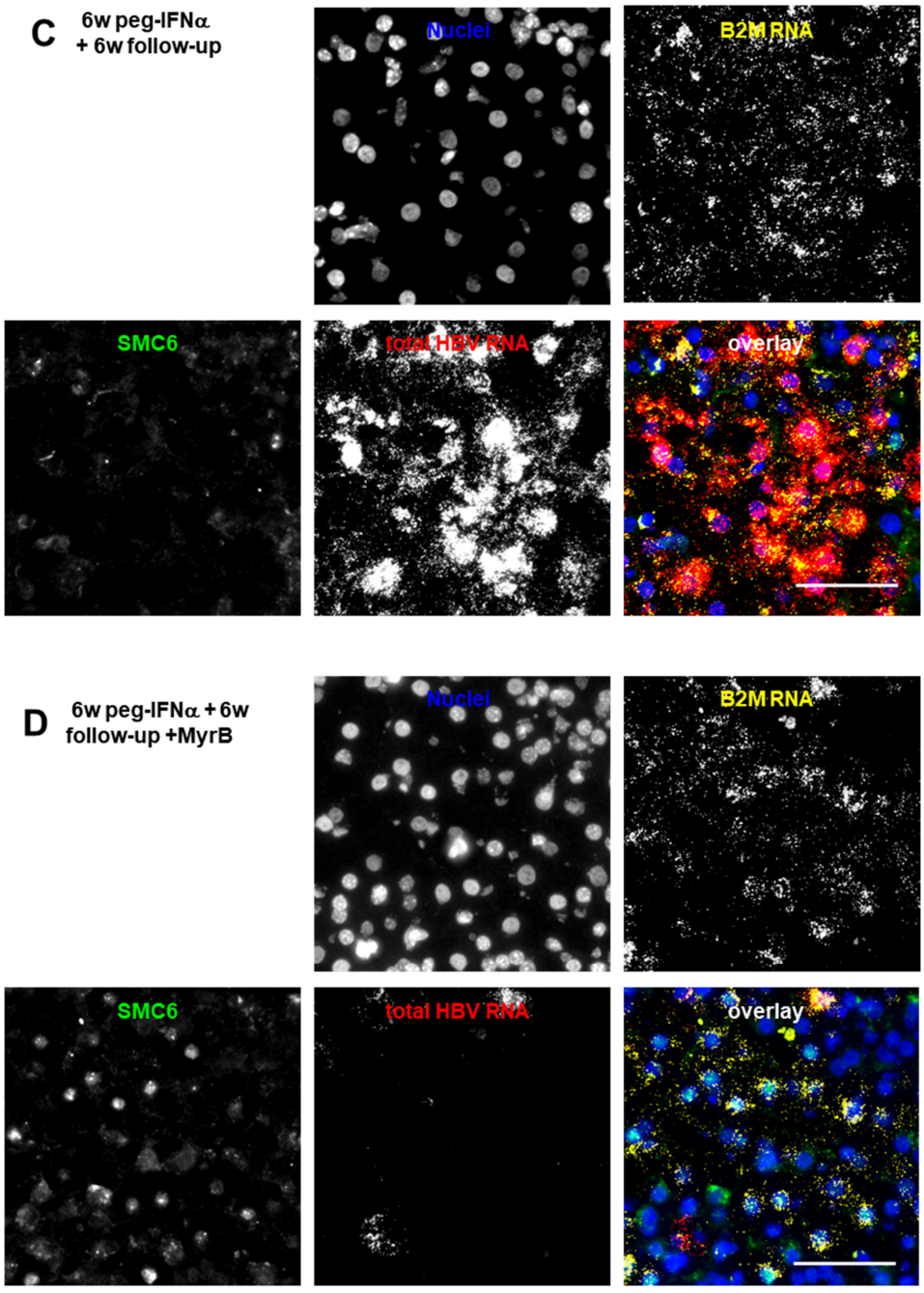
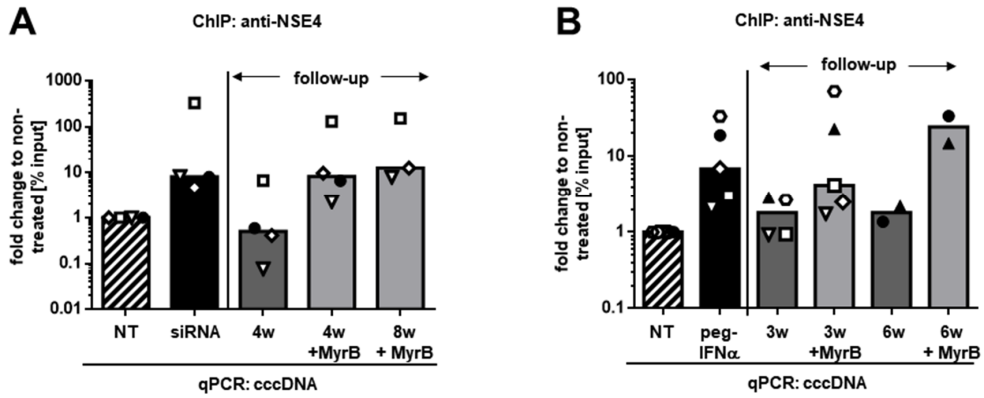


Suppl. Fig. 7: Peg-IFNα treatment leads to the reappearance of the SMC5/6 complex in hepatocytes negative for signs of active replication.

(A-D) Single channel photographs and overlays of RNA in situ hybridization for total HBV RNA combined with immunofluorescence staining for SMC6 protein. Shown are the same photographs as in figure 4 (representative pictures of one mouse from every treatment group as indicated at the left-hand side). Overlay pictures show nuclei stained with DAPI in blue, B2M RNA as a marker for human hepatocytes in aqua, SMC6 protein in green and total HBV RNA in red. Scale bar 50 µm.



Suppl. Fig. 8: Both siRNA and peg-IFNα treatment recruit the SMC5/6 complex to the cccDNA. (A, B) Chromatin immunoprecipitation assays were performed in two mice from every treatment group following siRNA (A) and peg-IFNα (B) treatment. Chromatin from liver tissue was precipitated with anti-NSE4 antibody and analyzed by cccDNA-selective qPCR. In one of the two mice from every group, the assay was performed in triplicates or quadruplicates, i.e. in separate chromatin preparations with distinct liver pieces. The mean of these measurements is also shown in figure 5C and D. Open symbols represent these replicate measurements and are shown alongside with the measurement from the second mouse (filled symbols) as shown in figure 5C and D. The form of the symbols indicates the measurements that were derived from ChIP assays performed in parallel, bars the median. NT, not treated.

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Supplementary references

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