1 **SUPPORTIVE INFORMATION**

2 **Supplementary Methods**

3 **Protein Expression and Purification.** The Y132A mutant HBV core protein N-terminal
4 domain (amino acids 1-149) of HBV genotype D strain adyw was cloned into a modified
5 pET23a Vector to produce a fusion protein with a C-terminal His6 tag and a preceding TEV
6 protease cleavage site (CoreND-Y132A). Cells were lysed by sonication and clarified lysate
7 was filtered, loaded onto a HiTrap Nickel Chelating column and the protein eluted using an
8 imidazole gradient. Eluted fractions containing the target protein were combined and
9 subjected to digestion by His6-tagged TEV protease at 4˚C resulting in the final protein with
10 residual residues KLENLYFQ on the C-terminus. Dialysis into 25 mM Tris pH 8.0, 100 mM
11 NaCl, 0.25% v/v glycerol, 2 mM TCEP was followed by a second HiTrap Nickel Chelating
12 column, to remove undigested material, cleaved tag, and TEV protease, while cleaved
13 CoreND-Y132A protein was collected in the flow-through. CoreND-Y132A protein was
14 concentrated to >15 mg/ml, then loaded onto a Sephacryl S-100 size exclusion column which
15 was equilibrated with 50 mM Tris pH 9.0, 2 mM DTT. Mutant protein eluted as a >95% pure
16 single peak which was concentrated to 10 mg/ml for crystallization experiments. The wild-
17 type and the Y132A coding sequences of HBV core protein (amino acids 1-149) of HBV
18 strain adyw were also cloned into a modified pET28 vector system which provides an N-
19 terminal His6-SUMO tag that could be removed by cleavage with Ulp-1 protease. The
20 purification protocols were similar as described above, and details are provided in
21 Supplemental Methods. The purification of the wild type protein included a urea based
22 denaturation and assembly step and a second denaturation as described originally by Zlotnick
23 et al. [1]. The wild-type and Y132A mutant HBV core proteins derived from the expression
24 with N-terminal His tags were used for biochemical experiments.

Electron Microscopy. Electron microscopy with HBV capsid formed from recombinant HBV core protein was performed as described [2]. Samples were adsorbed on 200 mesh copper grids coated with Formvar Carbon Film and stained with fresh 2% Uranyl Acetate. Samples were visualized on a FEI Technai T12 transmission electron microscope equipped with a 2Kx2K AMT MegaPLUS ES 4.0 CCD camera. Images were typically acquired with magnification of x105000.

Thermal Shift Analysis. Protein and ligand were incubated in assay buffer at a final concentration of 4 µM HBV core protein monomer, 25 µM ligands, 1% DMSO. Assay buffer was either 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM DTT (for CoreND-Y132A mutant dimer protein or assembled wild-type capsid) or 50 mM $NaHCO_3$, pH 9.2, 100 mM NaCl, 2mM DTT (for CoreND wild-type dimer protein). After one hour of incubation at room temperature, 25µL of each reaction and 2µL of spyro dye was added to each well of a 96-well plate in triplicate. Melting temperatures were measured from 20-100˚C over 70 minutes on a DNA Engine Opticon 2 Real-time cycler PCR (Biorad). Data were analyzed using Opticon Monitor II.

Analytical Size Exclusion Chromatography. High-performance liquid chromatography (HPLC) experiments were carried out at 4^{0}C using an Agilent 1100 Liquid Chromatography System outfitted with a Multi Wavelength Detector (MWD) and a Superdex 200 10/300 GL column. Chromatograms were evaluated with ChemStation (Agilent Technologies). 200 µg of purified CoreND protein were used with a mobile phase of 50 mM HEPES pH 7.5, 200 mM NaCl, 2 mM DTT. Gel Filtration Standards (BioRad) were run as part of the assay and protein molecular weights were determined based on linear regression analysis of those standards.

1 **Crystallization.** Mutant protein (CoreND-Y132A) at 10mg/ml in 50mM Tris pH 9.0, 2 mM
2 DTT was combined with reservoir solution containing 100 mM ammonium citrate/citric acid
3 pH 6.5, 9 % isopropanol, 10 % PEG 3350 supplemented with 10 % MPD at a 2:1 volume
4 ratio at 20°C. Thick hexagonal plates grew within 3 days. Crystals formed in space group C2
5 with cell dimensions a=152.6, b=88.2, c=102.25, β=131.5 and contain six capsid monomers
6 in the asymmetric unit. The crystals diffracted at a synchrotron to ~3.0Å; however when
7 soaked overnight with compound NR 010-001-E2, the diffraction improved to 1.95Å. Data
8 was collected at APS Beamline 21-IDf (LS-CAT). Data were reduced with xds, scaled with
9 xscale [3], solved by molecular replacement using 4BMG as a probe, refined in ccp4 using
10 refmac 5.0 [4], and rebuilt as needed with Coot [5]. The core protein was built through residue
11 Val148. Final data collection and refinement statistics are summarized in Table 1.

12 **Antiviral assays.** HepG2.2.15 cells (Fox Chase Cancer Center, Philadelphia, PA) were
13 maintained in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml
14 streptomycin, 2 mM L-glutamine and 380 μg/ml G418. Cells were seeded in 96-well plates at
15 a density of 40,000 cells/well, and treated with half-log serially diluted compounds at a final
16 DMSO concentration of 0.5%. Cells were incubated with compounds for three days, after
17 which medium was removed and fresh medium containing compounds was added to cells for
18 another three days. At day 6, cell culture supernatant was removed and treated with 2 units of
19 Turbo DNase (Invitrogen) at 37^{0}C for 60 minutes, followed by inactivation of the enzyme at
20 75^{0}C for 15 minutes. Encapsidated HBV DNA was released from the virions and covalently
21 linked HBV polymerase by incubating in lysis buffer with 2.5 μg proteinase K (Promega) and
22 detected by branched DNA assay using the QuantiGene assay kit and HBV DNA probes
23 according to manufacturer recommendation (Affymetrix). The mean background signal from
24 wells containing only culture medium was subtracted from all other samples, and percent
25 inhibition was calculated by normalizing to signals from HepG2.2.15 cells treated with 0.5%

DMSO. EC_{50} values were determined from the percent inhibition data obtained at different compound concentrations by non-linear fitting using Graphpad Prism software.

Transient transfection phenotyping. Plasmid DNA containing a 1.1x genotype B HBV genome under the control of a CMV promoter was previously cloned from serum of an HBV infected patient (Genbank AY220698, Fudan University, China) [6]. HBV core variants, W102G/R, I105V/L/T, T109S/M/I, and Y118F, were generated by site directed mutagenesis according to manufacturer recommendation (Agilent Technologies). The full length genome was sequenced to confirm that only the intended nucleotide changes were present in the final constructs. HepG2 cells (ATCC) maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, 10 μg/mL streptomycin, and 0.25 μg/mL of Fungizone were seeded in collagen coated 96-well plates at a density of 20,000 cells/well and allowed to attach overnight at 37°C and 5% CO_2. Cells were co-transfected with HBV (100 ng/well) and *Gaussia* luciferase reporter expression plasmids (10 ng/well) (Thermo Scientific) using the Lipofectamine LTX Plus transfection reagent. Transfection mixtures were removed the following day, and cells were treated with serially diluted compounds for three days at a final DMSO concentration of 0.5%, after which intracellular encapsidated HBV DNA was extracted from cells and levels of secreted *Gaussia* luciferase was determined from the medium using the *Gaussia* Flash Luciferase assay kit (Thermo Scientific). Briefly, cells were washed once with Dulbecco's phosphate-buffered saline and lysed with 0.33% NP-40. Nuclei were pelleted by centrifugation and supernatant containing HBV DNA was treated with Turbo DNase and S7 nuclease at 37^0C for 60 minutes, followed by inactivation of the enzymes at 75^0C for 15 minutes. Encapsidated HBV DNA was detected by branched DNA assay using the QuantiGene assay kit and HBV DNA probes as described above. To determine the replication competence of HBV core variants, the background corrected values for HBV DNA from the QuantiGene assay were normalized using the *Gaussia* luciferase

1 activity values in order to account for any differences in transfection efficiency. The
2 normalized HBV DNA values obtained from cells transfected with HBV variants were then
3 compared with those obtained from the wild type HBV transfection, with wild-type HBV
4 replication competence set at 100%.

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6 **Supplementary Table 1:** Conservation of amino acids within 5 Å of bound NVR 010-001-
7 E2 based on a sequence database of 2800 publicly available distinct HBV core protein
8 sequences across all HBV genotypes.

Protein chain[a]	Amino acid position	Amino acid	% Conservation
F	23	Phe	99.5
F	25	Pro	99.7
F	29	Asp	99.0
F	30	Leu	99.5
F	33	Thr	99.6
F	37	Leu	99.6
F	102	Trp	99.5
F	105	Ile	94.2
F	106	Ser	99.9
F	109	Thr	96.9
F	110	Phe	99.8
F	118	Tyr	99.2
F	122	Phe	99.7
F	139	Ile	99.9
F	140	Leu	99.9
F	141	Ser	99.8
A	124	Val	99.8
A	125	Trp	99.8
A	127	Arg	99.6
A	128	Thr	99.6
A	129	Pro	99.9
A	132	Ala	99.8
A	133	Arg	99.8
A	134	Pro	99.8

9 [a] Structural analysis based on the compound binding site between A and F monomers of the core
10 protein hexamer

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3 **Supplementary Table 2:** HBV core sequence analysis across 2800 public sequences of
4 genotypes A-H.

GT[a]	n[b]	Residue 105 (%)[c]	Reside 109 (%)[c]	Residue 102 (%)[c]	Reside 118 (%)[c]
A – H	2800	I(94.2), V(2.5), L(1.7), T(1.4) S/F/M/N	T(96.9), M(1.9) I/S/C/A/N	W(99.5) G/R	Y(99.2) F/N/E
A	355	I(94.0), T(3.7) S/L/V	T(98.3) M/A/S/C/I	W(98.6), R(1.1) G	Y(98.9), F(1.1)
B	588	I(94.0), L(2.9), V(2.7) T/M	T(94.9), M(2.7), I(1.2) S/C	W(99.8) G	Y(99.3) F
C	772	I(97.2), V(1.4), L(1.3) N	T(98.8), M(1.0) S	W(99.6) G/R	Y(99.9) E
D	790	I(94.6), V(4.8), T(2.5), L(1.7) F	T(95.6), M(2.8) S/I/A/N/C	W(99.8) G	Y(99.0) F/N
E	205	I(94.3), T(2.4), V(2.0) F/L	T(98.1), M(1.5) I	W(98.5), G(1.5)	Y(98.1), F(2.0)
F	54	I(96.2), L(3.8)	T(94.3), M(3.8), S(1.9)	W(98.2), G(1.9)	Y(98.2), F(1.9)
G	16	I(100)	T(100)	W(100)	Y(100)
H	20	I(100)	T(100)	W(100)	Y(100)

5 [a] HBV genotype
6 [b] Number of sequences analyzed
7 [c] Amino acids reported in the sequence database at this position; brackets show the percentage prevalence of a
8 particular amino acid at this position, if it exceeded 1%

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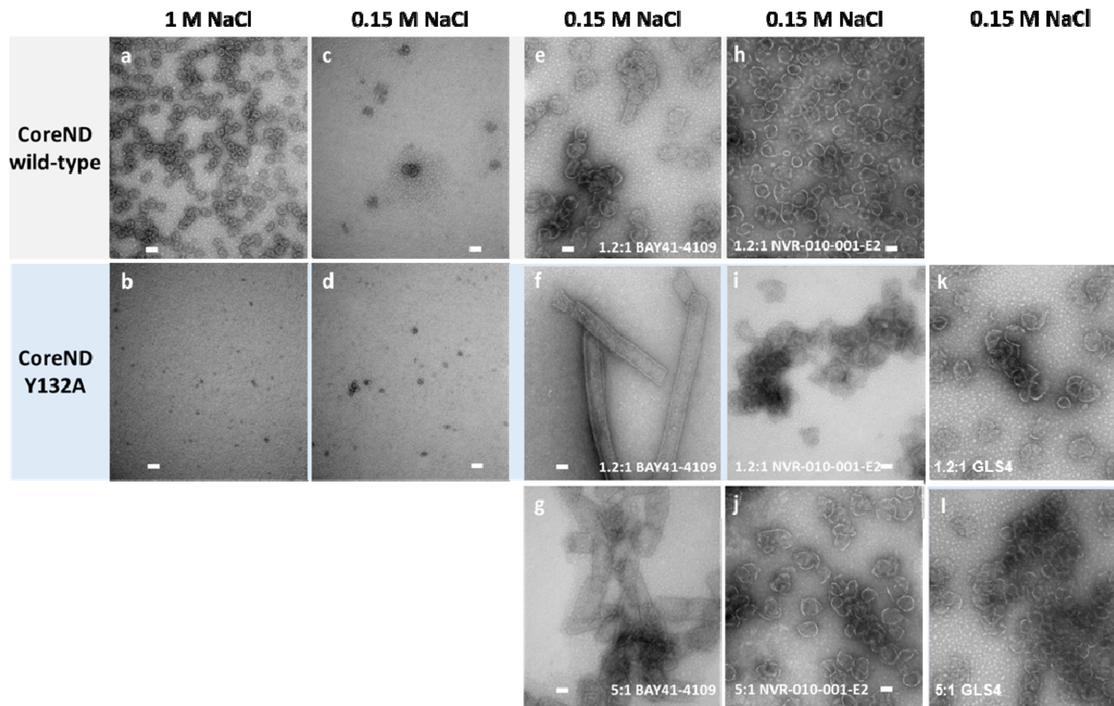

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2 **Supplementary Figure 1**

3 Electron microscopy of wildtype HBV core protein (top row) and Y132A mutant HBV core protein at
4 10 µM monomer concentration; **a),b)** Protein in the presence of 1M NaCl; **c),d)** Protein in the
5 presence of 0.15M NaCl; **e),f),h),i),k)** Protein in the presence of 0.15M NaCl and 1.2-fold excess of
6 either BAY 41-4109, NVR-010-001-E2 or GLS4 as indicated; **g),j),l)** Protein in the presence of
7 0.15M NaCl and 5-fold excess of either BAY 41-4109, NVR-010-001-E2 or GLS4 as indicated.

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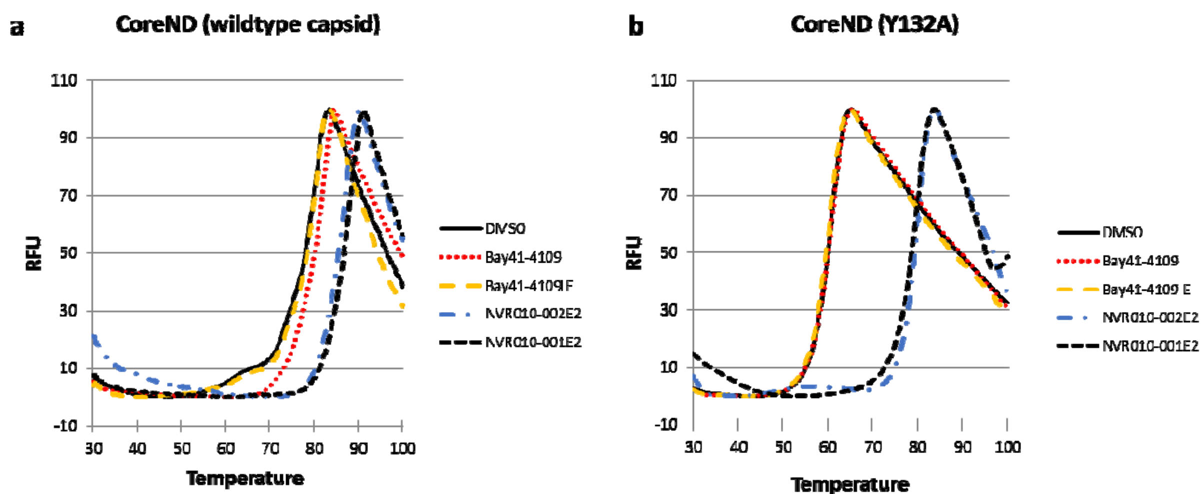

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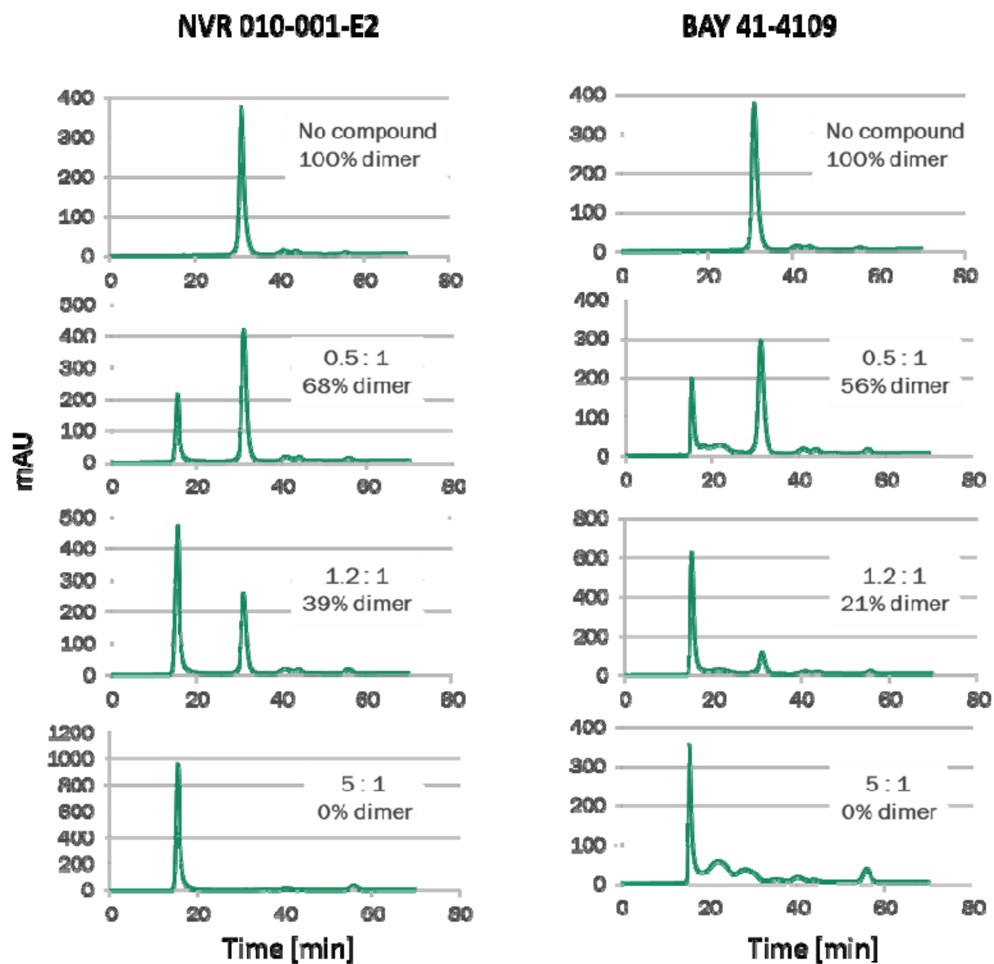
2 **Supplementary Figure 2**

3 Core inhibitors can thermostabilize preformed HBV capsids and Y132A mutant HBV core protein. **a)**
4 Thermal shift assay using wild-type HBV capsid formed from CoreND protein; **b)** thermal shift assay
5 using CoreND-Y132A protein. Temperature induced unfolding in the absence of compound (DMSO,
6 black, solid line), in the presence of BAY 41-4109-IE (orange, wide dashed line), BAY 41-4109 (red,
7 dotted line), GLS4 (NVR-010-002-E2, blue, dotted-dashed line), NVR-010-001-E2 (black, dashed
8 line).



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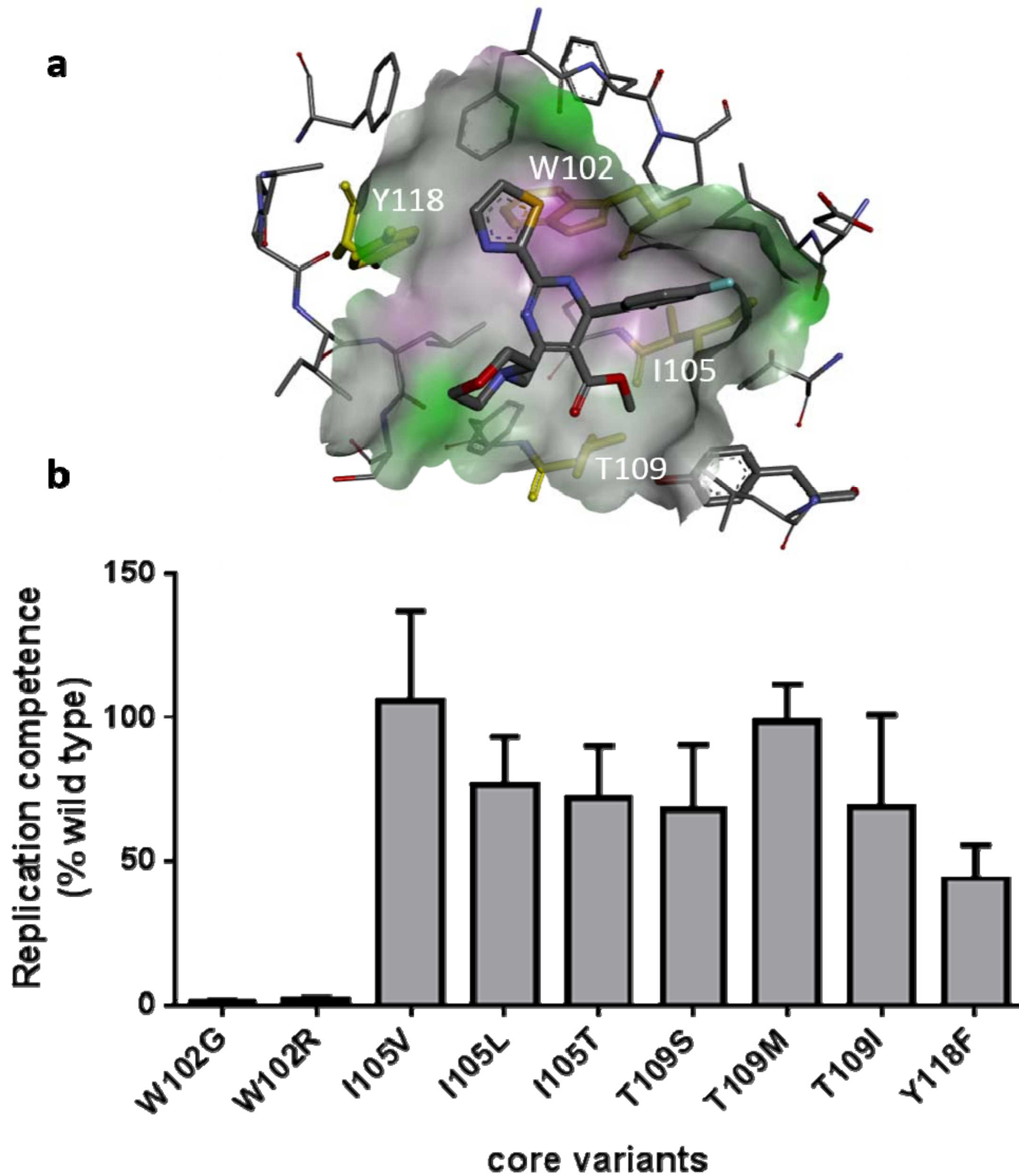
2 **Supplementary Figure 3**

3 Analytical size exclusion chromatography (anSEC) of CoreND-Y132A protein alone or in the
4 presence of different ratios of core inhibitors NVR 010-001-E2 or BAY 41-4109 relative to CoreND-
5 Y132A protein monomer as indicated on the graphs. In the presence of 5:1 compound to protein
6 monomer ratio, all of the dimer is assembled into larger structures that elute early from the size
7 exclusion column.



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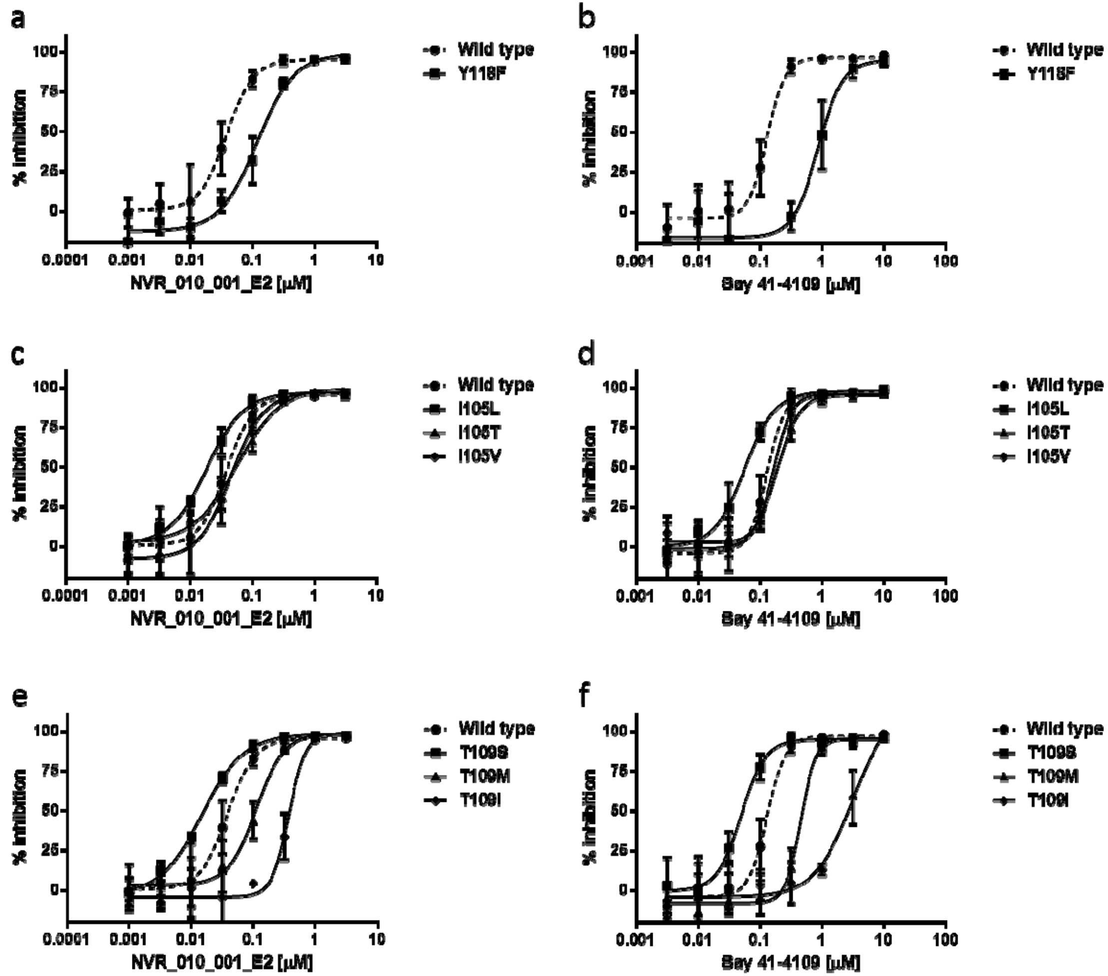
2 **Supplementary Figure 4**

3 Evaluation of core variants at polymorphic sites near the bound ligand. **a)** Location of the four amino
4 acids (colored yellow) with variants occurring at >1% frequency in any genotype (A-H). **b)**
5 Replication competence of naturally occurring core variants determined by transient transfection
6 studies in HepG2 cells. Intracellular encapsidated HBV DNA levels were normalized for transfection
7 efficiency using secreted Gaussia Luciferase and compared to DNA levels obtained with wild type
8 HBV. Results shown are mean values and Standard Deviation error bars, determined from at least
9 three independent studies.



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2 **Supplementary Figure 5**

3 Effect of HBV core protein variants on antiviral activities of NVR_010_001_E2 **(a c, e)** or BAY 41-
4 4109 **(b, d, f)**. HepG2 cells transfected with HBV plasmids were incubated with increasing
5 concentrations of core modulators for 3 days and intracellular encapsidated HBV DNA levels were
6 measured and normalized to untreated controls. Data points shown are mean values and error bars are
7 Standard Deviations determined from at least three independent studies..

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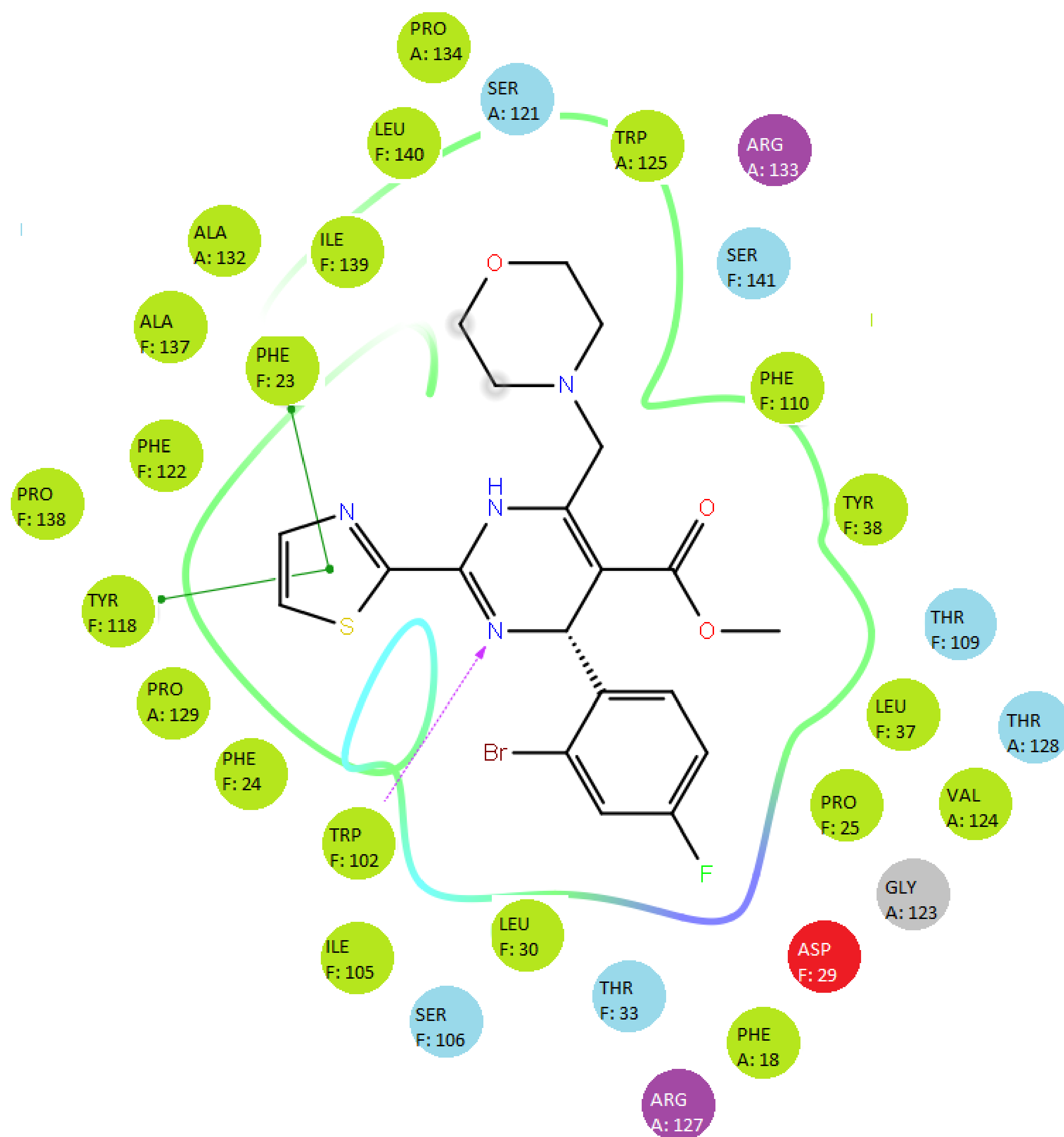

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2 **Supplementary Figure 6**

3 2D view of the interactions of NVR 010-001-E2 with the binding pocket formed by A (lid) and F
4 (contact domain) core protein monomers. Hydrophobic residues are shown in green, polar in blue,
5 negatively charged in red, positively charged in purple and glycine in grey. Hydrogen bond to Trp 102
6 is shown with a purple arrow and aromatic stacking with green lines

7

References

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