Manuscript Details

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Title	Interplay between SIRT1 and hepatitis B virus X protein in the activation of viral transcription
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Abstract

Hepatitis B virus (HBV) genome is organized into a minichromosome known as covalently closed circular DNA (cccDNA), which serves as the template for all viral transcripts. SIRT1 is an NAD+-dependent protein deacetylase which activates HBV transcription by promoting the activity of cellular transcription factors and coactivators. How SIRT1 and viral transactivator X protein (HBx) might affect each other remains to be clarified. In this study we show synergy and mutual dependence between SIRT1 and HBx in the activation of HBV transcription. All human sirtuins SIRT1 through SIRT7 activated HBV gene expression. The steady-state levels of SIRT1 protein were elevated in HBV-infected liver tissues and HBV-replicating hepatoma cells. SIRT1 interacted with HBx and potentiated HBx transcriptional activity on precore promoter and covalently closed circular DNA (cccDNA) likely through a deacetylase-independent mechanism, leading to more robust production of cccDNA, pregenomic RNA and surface antigen. SIRT1 and HBx proteins were more abundant when both were expressed. SIRT1 promoted the recruitment of HBx as well as cellular transcriptional factors and coactivators such as PGC-1α and FXRα to cccDNA. Depletion of SIRT1 suppressed HBx recruitment. On the other hand, SIRT1 recruitment to cccDNA was compromised when HBx was deficient. Whereas pharmaceutical agonists of SIRT1 such as resveratrol activated HBV transcription, small-molecule inhibitors of SIRT1 including sirtinol and Ex527 exhibited anti-HBV activity. Taken together, our findings revealed not only the interplay between SIRT1 and HBx in the activation of HBV transcription but also new strategies and compounds for developing antivirals against HBV.

Keywords	hepatitis B virus; sirtuin; HBx; cccDNA; SIRT1
Taxonomy	Viral Hepatitis, Protein-DNA Interactions, Viral Transcription, Protein-Protein Interaction

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Interplay between SIRT1 and hepatitis B virus X protein in the activation of viral transcription

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Figure legends

Fig. 1. All mammalian sirtuins positively regulated HBV transcription. (A) Verification of the expression of FLAG-tagged sirtuins in HepG2-NTCP cells. (B) cccDNA levels in sirtuin-expressing cells. HepG2-NTCP cells were transfected with pHBV1.3D and sirtuin plasmids. Cells were harvested 48 h post-transfection. Nuclear DNA was isolated and treated sequentially with SacI restriction endonuclease and plasmid-safe ATP-dependent DNase. Treated fraction was analyzed for cccDNA by qPCR. GAPDH transcript was amplified from untreated cytoplasmic fraction for normalization. Results are representation of three independent experiments and error bars indicate SD. The difference between control and SIRT7 groups is statistically significant ($p = 0.014$). N.D.: not detected. (C) pgRNA levels in sirtuin-expressing cells. Total RNA was extracted and cDNA was synthesized. RT-qPCR was performed to analyze the relative levels of pgRNA. The difference between control and SIRT7 groups is statistically significant ($p = 0.024$). (D) Levels of extracellular HBsAg in sirtuin-expressing cells. Culture media were centrifuged to remove debris and HBsAg was assayed by ELISA. Relative HBsAg level in cells transfected with pHBV1.3D alone was taken as 1. The differences between control and SIRT6 groups is statistically significant ($p = 0.0033$).

Fig. 2. Elevation of SIRT1 protein expression in HBV-infected human liver tissues and HBV-replicating HepG2-NTCP cells. (A) Steady-state levels of SIRT1 protein in HBV^- and HBV^+ human liver tissues. Western blotting was performed with rabbit anti-SIRT1 and mouse anti-β-actin antibodies. (B) Steady-state levels of SIRT1 protein in HepG2-NTCP cells transfected with increasing amounts of pHBV1.3D and in HepG2.2.15 cells. Cells were harvested 48 h post-transfection for Western blot analysis.

Fig. 3. SIRT1 augmented HBx-induced activation of HBV transcription. (A, B) Levels of pgRNA and extracellular HBsAg in HBV-infected cells. HepG2-NTCP cells were infected with HBV at a multiplicity of genome equivalents of 100. After 6 days cells were co-transfected with expression plasmids for SIRT1 and HBx for another 2 days. pgRNA was analyzed by RT-qPCR (A). Culture media were centrifuged to remove debris and HBsAg was assayed by ELISA (B). Relative pgRNA or HBsAg level in cells infected with HBV alone was taken as 1. The differences between bars 2 and 3 ($p = 0.008656$ for panel A and $p = 0.000079$ for panel B) as well as between bars 3 and 5 ($p = 0.0395$ for panel A and $p = 0.000414$ for panel B) are statistically significant. N.D.: not detected. (C, D) cccDNA levels in transfected cells. Cells were co-transfected with pHBV1.3D (C) or its HBx-deficient mutant pHBV-X^- (D) and expressing plasmid for SIRT1 wild-type (WT) or its H363Y. Either HBx or PGC-1α was also expressed. The differences between bars 2 and 3 ($p = 0.0053$) and between bars 5 and 7 ($p = 0.0013$) in panel C as well as between bars 6 and 8 in panel D ($p = 0.013$) are statistically significant. The differences between bars 2 and 4 ($p = 0.34$) and between bars 8 and 10 ($p = 0.73$) in panel C are statistically not significant (n.s.). (E, F) pgRNA levels in transfected cells. The differences between bars 2 and 3 ($p = 0.0044$) and between bars 5 and 7 ($p = 0.00006$) in panel E as well as between bars 6 and 8 in panel F ($p = 0.0015$) are statistically significant. The differences between bars 2 and 4 ($p = 0.30$) and between bars 8 and 10 ($p = 0.27$) in panel E are statistically not significant (n.s.). (G) Levels of extracellular HBsAg in transfected cells. The differences between bars 2 and 3 ($p = 0.000019$) and between bars 5 and 7 ($p = 0.000018$) are statistically significant. The differences between bars 2 and 4 ($p = 0.074$) and between bars 8 and 10 ($p = 0.10$) are statistically not significant (n.s.). (H, I) SIRT1 and HBx mRNA levels. Cells were harvested 48 h post-transfection for RT-qPCR analysis. The differences between bars

1 and 2 (p = 0.00003) and between bars 4 and 5 (p = 0.00014) in panel H are statistically significant. The differences between bars 1 and 2 (p = 0.41) and between bars 4 and 5 (p = 0.07) are statistically not significant (n.s.). (J) Activation of HBV preC promoter. HepG2-NTCP cells transfected with the indicated plasmids were harvested at 36 h after transfection. Dual luciferase assays were performed. The luciferase activity was normalized to RLuc readings and expressed as fold activation. The difference between bars 1 and 2 (p = 0.046) is statistically significant. (K) SIRT1 recruitment to preC promoter. ChIP was performed with rabbit anti-SIRT1 and the preC promoter-specific sequence was analyzed by qPCR. Results represent relative recruitment measured in arbitrary units (AU). The difference between bars 2 and 3 (p = 0.000087) is statistically significant. N.D.: not detected.

Fig. 4. SIRT1 facilitated recruitment of HBx, PGC-1α and FXRα to cccDNA. HepG2-NTCP cells were co-transfected with pHBV1.3D and expression plasmid for FLAG-SIRT1 for 48 h. ChIP was performed to precipitate SIRT1-, HBx-, PGC-1α- and FXRα-bound DNA complex using anti-FLAG (A), anti-HBx (B), anti-PGC-1α (C) and anti-FXRα (D). The differences between bars 2 and 3 are statistically significant (A: p = 0.0011; B: p = 0.0018; C: p = 0.0006; D: p = 0.0018). N.D.: not detected.

Fig. 5. Depleting SIRT1 suppressed HBV transcription and HBx recruitment to cccDNA. (A) Depletion of endogenous SIRT1 in HepG2-NTCP cells and HepG2.2.15 cells by siRNAs. Cells were transfected with two independent SIRT1-targting siRNAs (siSIRT1-a and siSIRT1-b) or siNC (negative control) at a concentration of 100 nM for 48 h. The transfection efficiency of siRNAs was estimated to be 75%. Western blotting was performed with rabbit anti-SIRT1 and mouse anti-β-actin antibodies. (B) Decrease

of cccDNA levels in SIRT1-depleted HepG2.2.15 cells. The difference between bars 1 and 2 ($p = 0.049$) is statistically significant. (C) Knockdown of SIRT1 suppressed pgRNA expression in pHBV1.3D-transfected HepG2-NTCP cells. The difference between bars 2 and 4 ($p = 0.0065$) is statistically significant. (D-F) Depletion of SIRT1 perturbed HBx, PGC-1α and FXRα recruitment to cccDNA. HepG2.2.15 cells were transfected with the indicated siRNAs for 48 h. The differences between bars 1 and 3 are statistically significant(D: $p = 0.0002$; E: $P = 0.00003$; F: $p = 0.000015$).

Fig. 6. Functional interplay between SIRT1 and HBx. (A) Co-immunoprecipitation of SIRT1 and HBx from HepG2-NTCP cells. Cells were co-transfected with the indicated expression plasmids and harvested at 48 h after transfection. Immunoprecipitation (IP) was performed with anti-HA. Precipitates and input (5%) were analyzed by Western blotting with mouse anti-FLAG, mouse anti-HA, mouse anti-β-actin and rabbit anti-AcK. Bands for immunoglobulin heavy and light chains are indicated by "#" and "Δ", respectively. (B) Abundance of SIRT1 and HBx proteins when they were co-expressed. Western blotting was performed with mouse anti-FLAG, anti-HA and anti-β-actin. (C) HBx protein expression in HepG2-NTCP cells transfected with pHBV1.3D and SIRT1 expression plasmid. (D) SIRT1 protein expression in HepG2-NTCP cells transfected with pHBV1.3D (pHBV-WT) or its HBx-deficient mutant pHBV-X^-. Western blotting was performed with rabbit anti-SIRT1, rabbit anti-HBx and mouse anti-β-actin. (E, F) Loss of HBx suppressed the SIRT1 recruitment to cccDNA and preC promoter. HepG2-NTCP cells were co-transfected with the indicated plasmids. ChIP was performed to precipitate SIRT1-DNA complex. The differences between bars 2 and 3 are statistically significant, with P values equal to 0.0087 (E) and 0.0039 (F), respectively.

Fig. 7. Influence of SIRT1 modulators on HBV transcription. (A) Decline of cccDNA levels in HepG2.2.15 cells treated with SIRT1 inhibitors (SIRT1*i*). HepG2.2.15 cells were treated with increasing doses (25, 50 and 75 μM) of sirtinol and Ex527 for 36 h. The difference between bars 2 and 4 is statistically significant ($p = 0.018$). (B) Decline of pgRNA expression in HepG2.2.15 cells treated with SIRT1*i*. The difference between bars 2 and 4 is statistically significant ($p = 0.0036$). (C) Time course of HBsAg detection in culture media of HBV-infected HepG2-NTCP cells treated with SIRT1*i*. HepG2-NTCP cells were infected with HBV at a multiplicity of genome equivalents of 100. After 8 days cells were treated with 50 μM of sirtinol or Ex527 for 48 h. Culture supernatants were collected every 12 h for ELISA analysis of HBsAg. The differences between DMSO and Sirtinol groups ($p = 0.0045$) and between DMSO and Ex527 groups ($p = 0.041$) are statistically significant. (D) Elevation of cccDNA levels in HBV-infected HepG2-NTCP cells treated with 25, 50 and 75 μM of resveratrol (Res). Difference between bars 2 and 3 is statistically significant ($p = 0.0081$). (E) Increased pgRNA levels in HBV-infected HepG2-NTCP cells treated with resveratrol (Res). The difference between bars 2 and 3 is statistically significant ($p = 0.0013$).

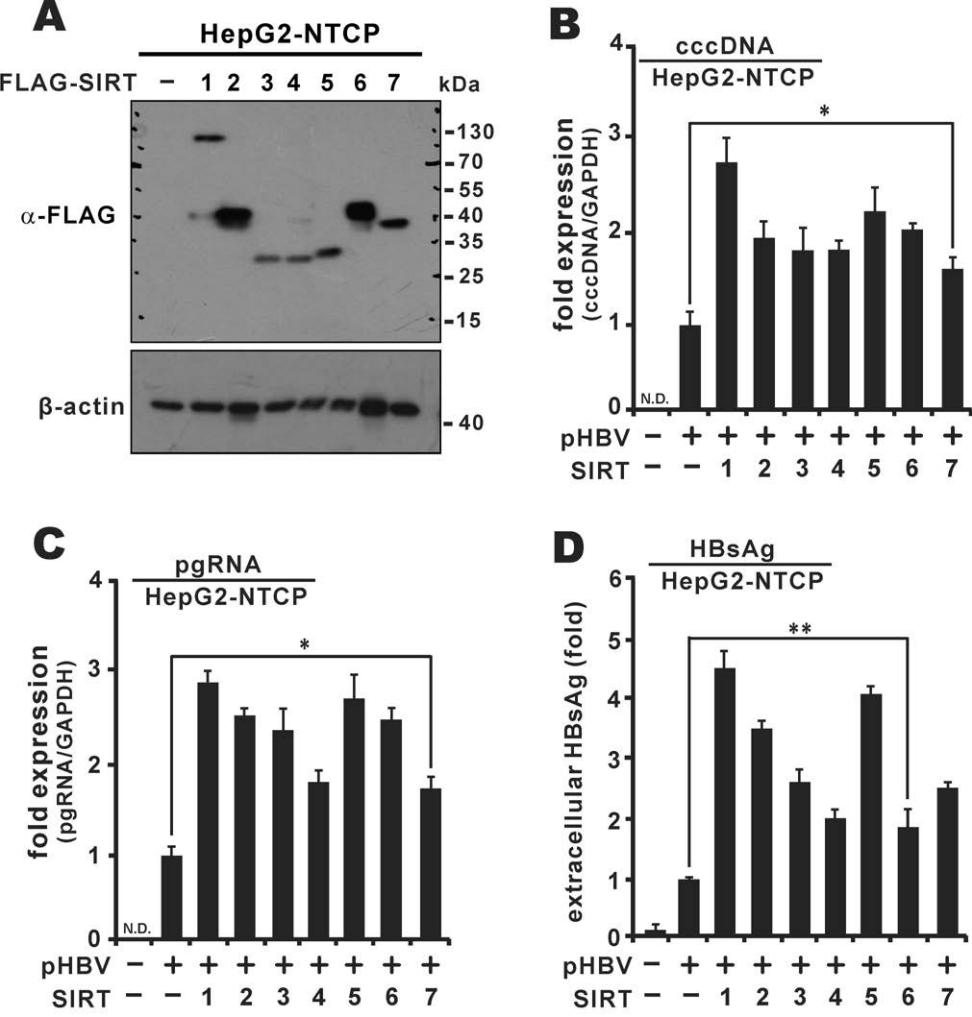
A
HepG2-NTCP
FLAG-SIRT – 1 2 3 4 5 6 7 kDa
130
70
55
40
35
25
15
α-FLAG
β-actin
40
B
cccDNA
HepG2-NTCP
fold expression
(cccDNA/GAPDH)
*
N.D.
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7
C
pgRNA
HepG2-NTCP
fold expression
(pgRNA/GAPDH)
*
N.D.
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7
D
HBsAg
HepG2-NTCP
extracellular HBsAg (fold)
**
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7

Figure 1

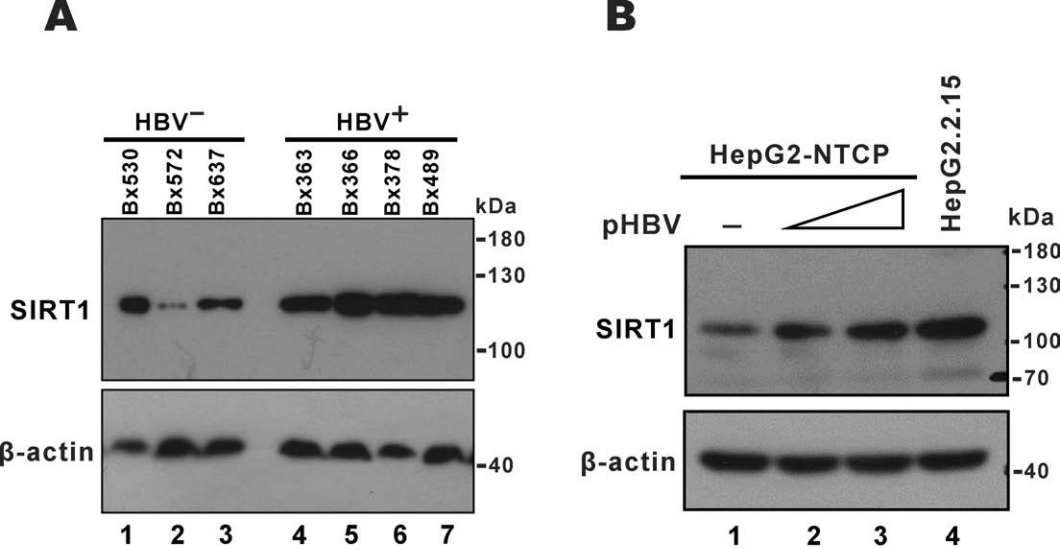
A
HBV−
HBV+
Bx530
Bx572
Bx637
Bx363
Bx366
Bx378
Bx489
kDa
SIRT1
-180
-130
-100
β-actin
-40
1 2 3 4 5 6 7
B
HepG2-NTCP
HepG2.2.15
pHBV
–
kDa
SIRT1
-180
-130
-100
-70
β-actin
-40
1 2 3 4

Figure 2

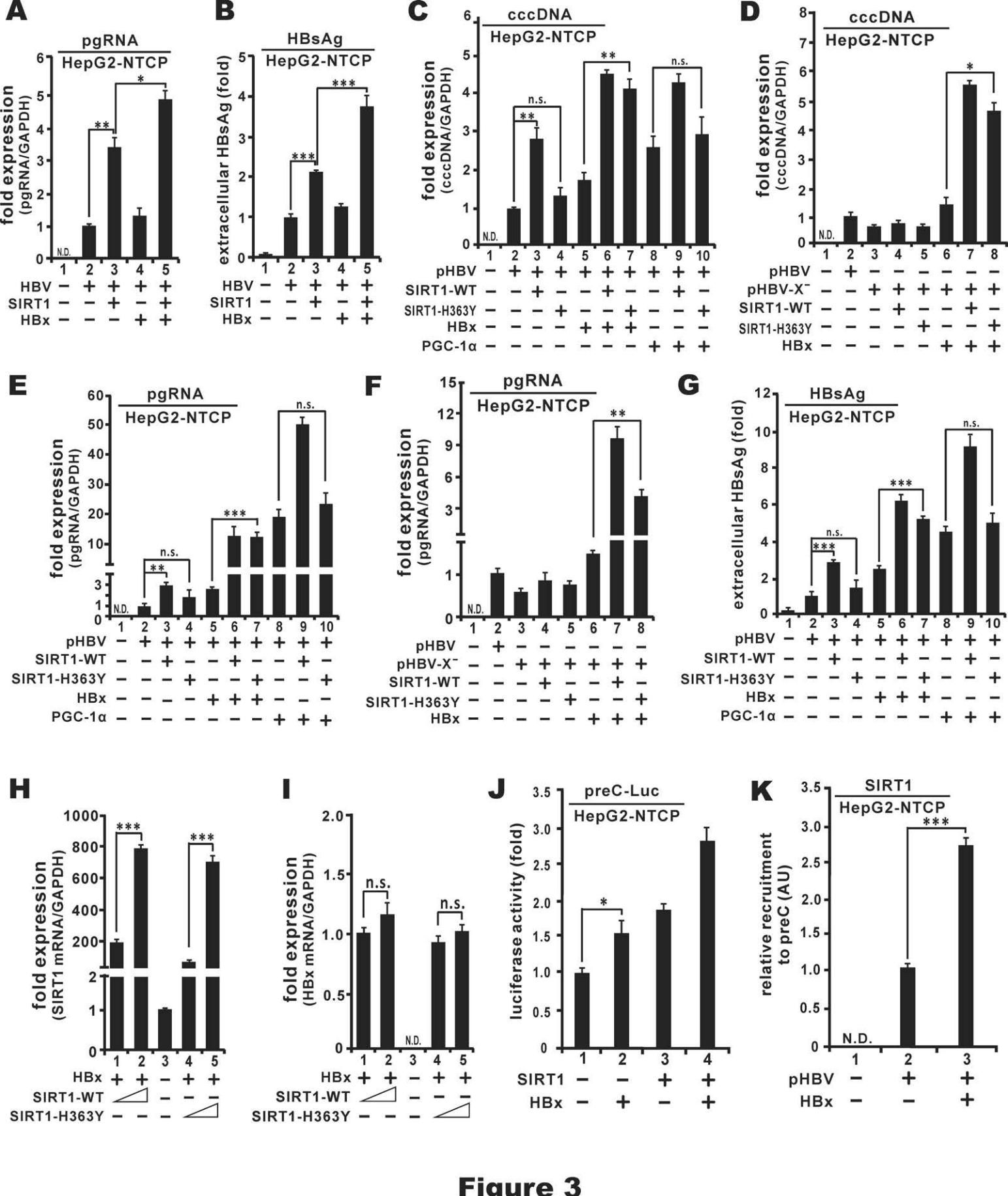
A
pgRNA
HepG2-NTCP
fold expression (pgRNA/GAPDH)
N.D.
HBV
SIRT1
HBx
B
HBsAg
HepG2-NTCP
extracellular HBsAg (fold)
C
cccDNA
HepG2-NTCP
fold expression (cccDNA/GAPDH)
n.s.
pHBV
SIRT1-WT
SIRT1-H363Y
HBx
PGC-1α
D
cccDNA
HepG2-NTCP
pHBV-X⁻
E
pgRNA
HepG2-NTCP
F
pgRNA
HepG2-NTCP
G
HBsAg
HepG2-NTCP
H
fold expression (SIRT1 mRNA/GAPDH)
I
fold expression (HBx mRNA/GAPDH)
J
preC-Luc
HepG2-NTCP
luciferase activity (fold)
K
SIRT1
HepG2-NTCP
relative recruitment to preC (AU)

Figure 3

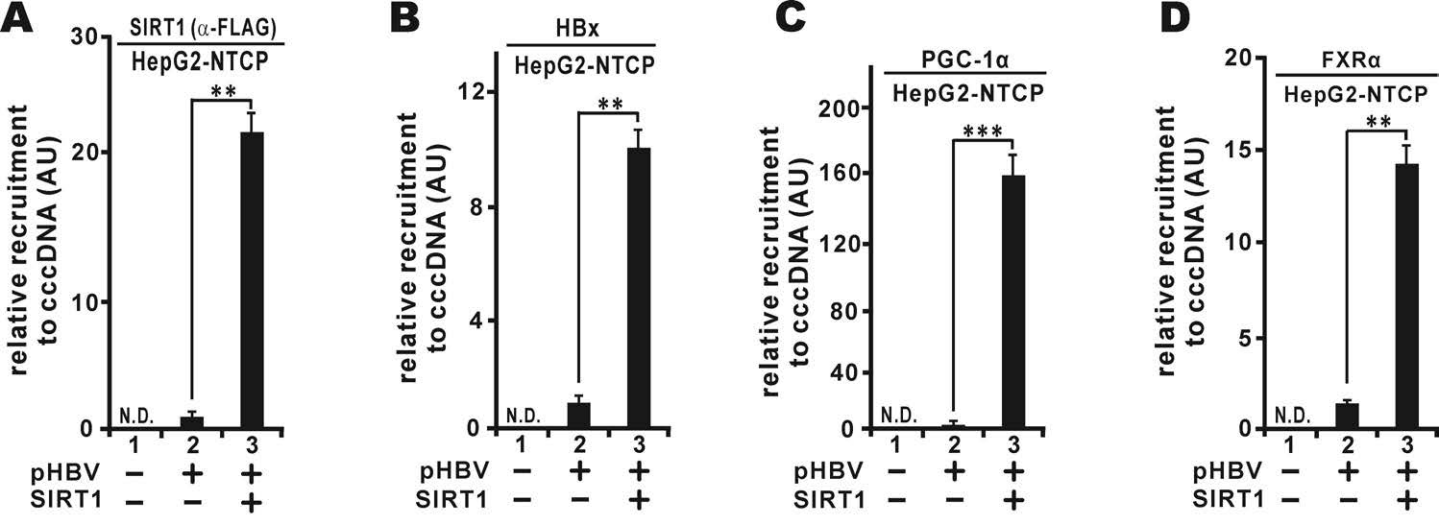
A
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to cccDNA (AU)
30
20
10
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
B
HBx
HepG2-NTCP
relative recruitment to cccDNA (AU)
12
8
4
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
C
PGC-1α
HepG2-NTCP
relative recruitment to cccDNA (AU)
200
160
120
80
40
0
***
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
D
FXRα
HepG2-NTCP
relative recruitment to cccDNA (AU)
20
15
10
5
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +

Figure 4

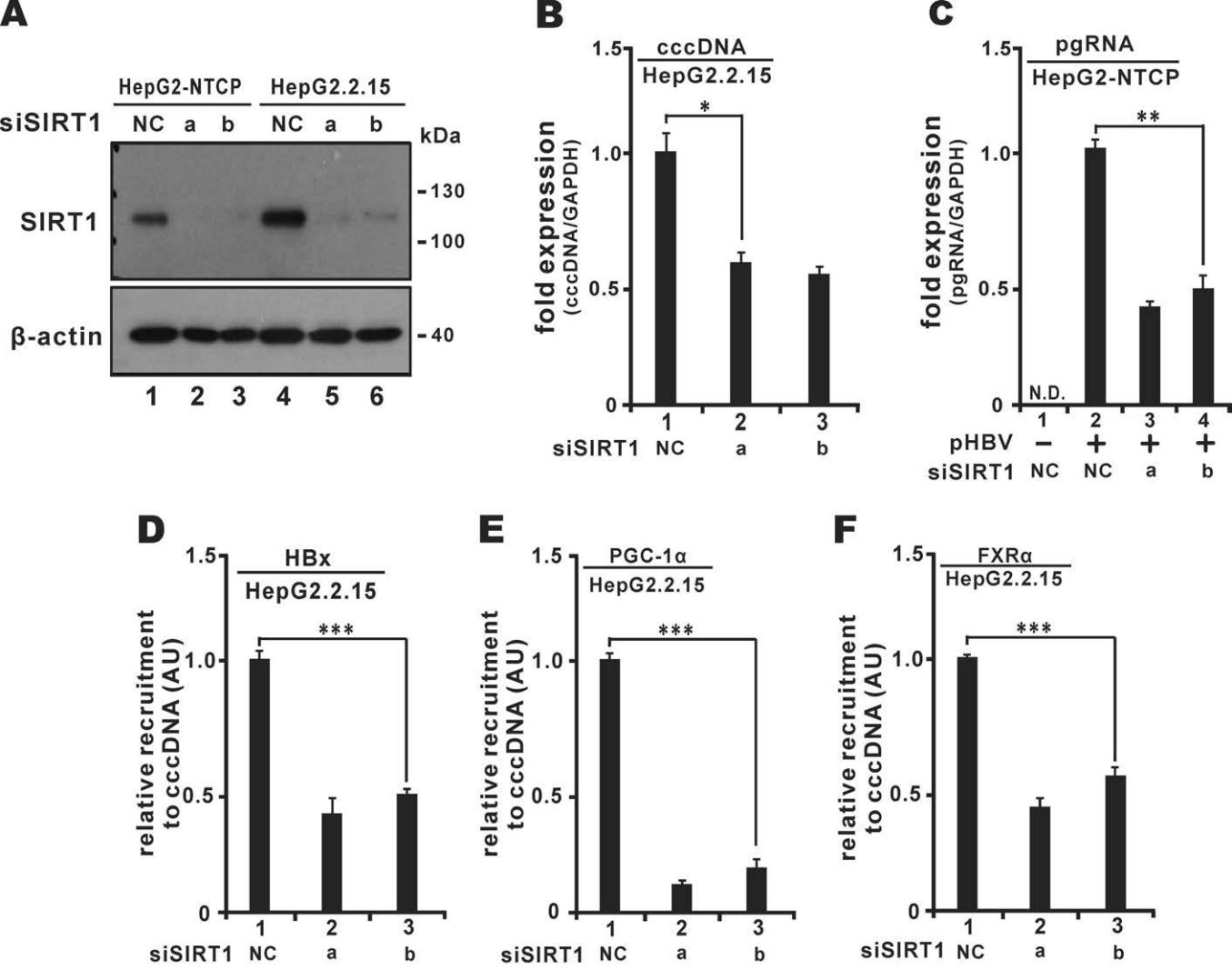
A
HepG2-NTCP
HepG2.2.15
siSIRT1
NC a b NC a b
kDa
SIRT1
β-actin
130
100
40
1 2 3 4 5 6
B
cccDNA
HepG2.2.15
fold expression
(cccDNA/GAPDH)
*
siSIRT1 NC a b
C
pgRNA
HepG2-NTCP
fold expression
(pgRNA/GAPDH)
**
N.D.
pHBV – + + +
siSIRT1 NC NC a b
D
HBx
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b
E
PGC-1α
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b
F
FXRα
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b

Figure 5

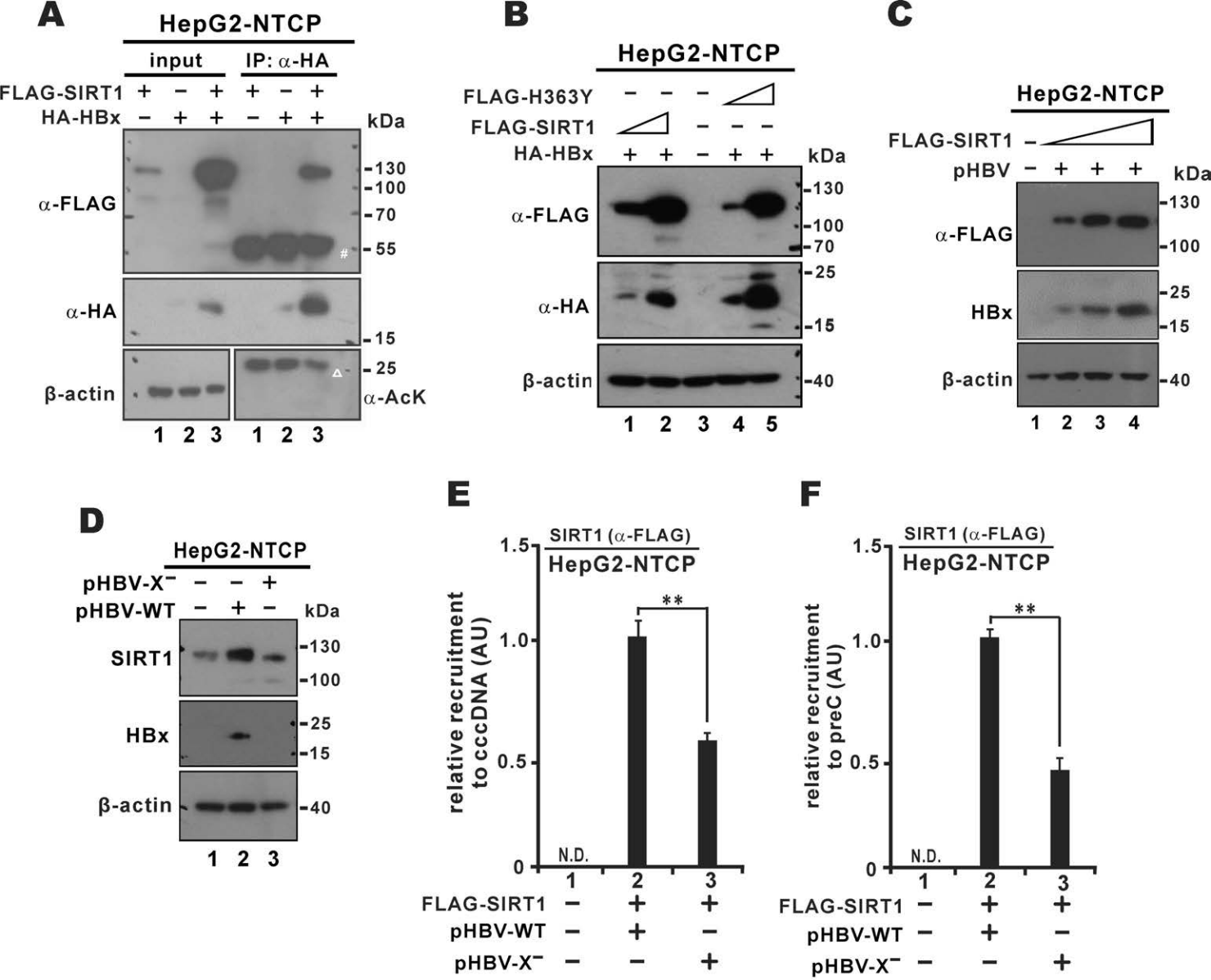
A
HepG2-NTCP
input
IP: α-HA
FLAG-SIRT1 + − + + − +
HA-HBx − + + − + +
kDa
α-FLAG
α-HA
β-actin
α-AcK
130
100
70
55
15
25
1 2 3 1 2 3
B
HepG2-NTCP
FLAG-H363Y
FLAG-SIRT1
HA-HBx + + − + +
kDa
α-FLAG
α-HA
β-actin
130
100
70
25
15
40
1 2 3 4 5
C
HepG2-NTCP
FLAG-SIRT1
pHBV − + + +
kDa
α-FLAG
HBx
β-actin
130
100
25
15
40
1 2 3 4
D
HepG2-NTCP
pHBV-X⁻ − − +
pHBV-WT − + −
kDa
SIRT1
HBx
β-actin
130
100
25
15
40
1 2 3
E
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to cccDNA (AU)
1.5
1.0
0.5
0
**
N.D.
1 2 3
FLAG-SIRT1 − + +
pHBV-WT − + −
pHBV-X⁻ − − +
F
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to preC (AU)
1.5
1.0
0.5
0
**
N.D.
1 2 3
FLAG-SIRT1 − + +
pHBV-WT − + −
pHBV-X⁻ − − +

Figure 6

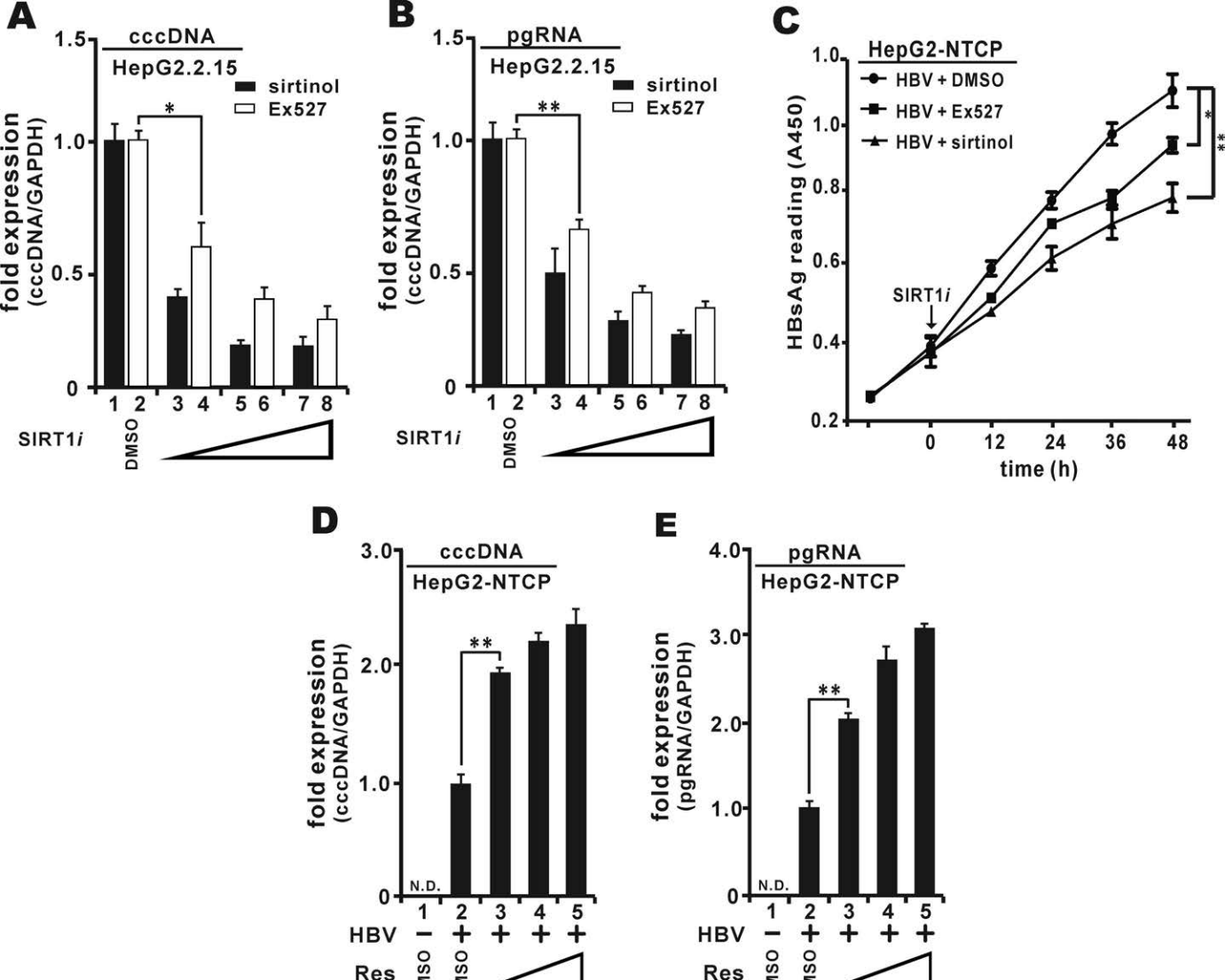
A
cccDNA
HepG2.2.15
sirtinol
Ex527
fold expression
(cccDNA/GAPDH)
SIRT1i
DMSO
B
pgRNA
HepG2.2.15
C
HepG2-NTCP
HBV + DMSO
HBV + Ex527
HBV + sirtinol
HBsAg reading (A450)
time (h)
D
cccDNA
HepG2-NTCP
N.D.
HBV
Res
E
pgRNA
HepG2-NTCP
(pgRNA/GAPDH)

Figure 7

Interplay between SIRT1 and hepatitis B virus X protein in the activation of viral transcription

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Supplementary material

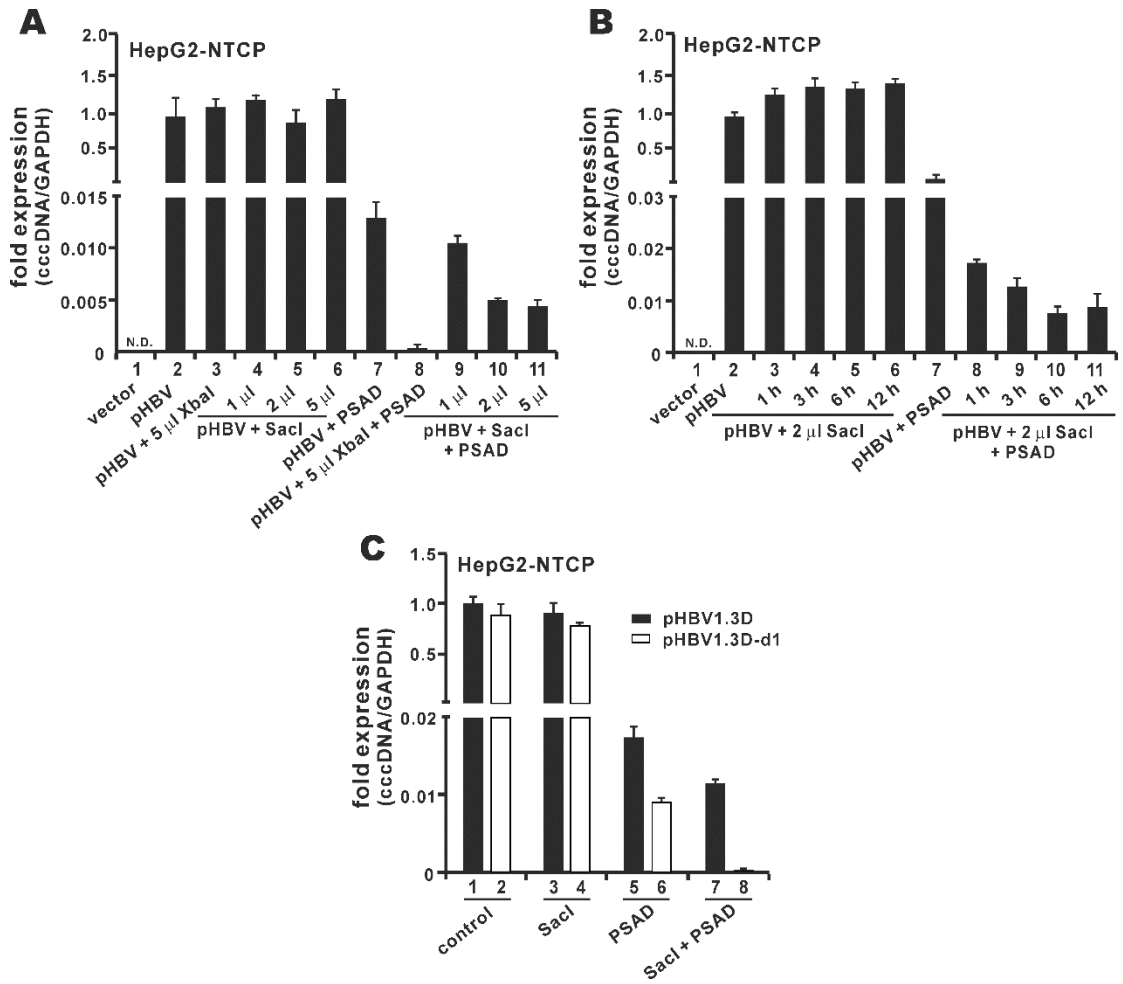

Fig. S1. cccDNA detection in pHBV1.3D-transfected HepG2-NTCP cells. Cells were transfected with pHBV1.3D or its disrupted version pHBV1.3D-d1 and harvested 48 h after transfection for cccDNA detection by RT-qPCR. (A) Isolated nuclear DNA (200 ng) was digested with XbaI restriction enzyme (5 µl) or increasing amounts of SacI restriction enzyme (1, 2 and 5 µl) for 2 h at 37 °C and subsequently with plasmid-safe ATP-dependent DNase (PSAD). Whereas XbaI cuts the HBV genome at one single site so that no cccDNA can be formed, there is only one single SacI site outside of the HBV genome in pHBV1.3D. (B) Isolated nuclear DNA (200 ng) was digested with SacI (2 µl) for 1, 3, 6 and 12 h at 37 °C and subsequently with PSAD. (C) Isolated nuclear DNA (200 ng) was digested with SacI (2 µl) for 6 h at 37 °C and subsequently with PSAD.

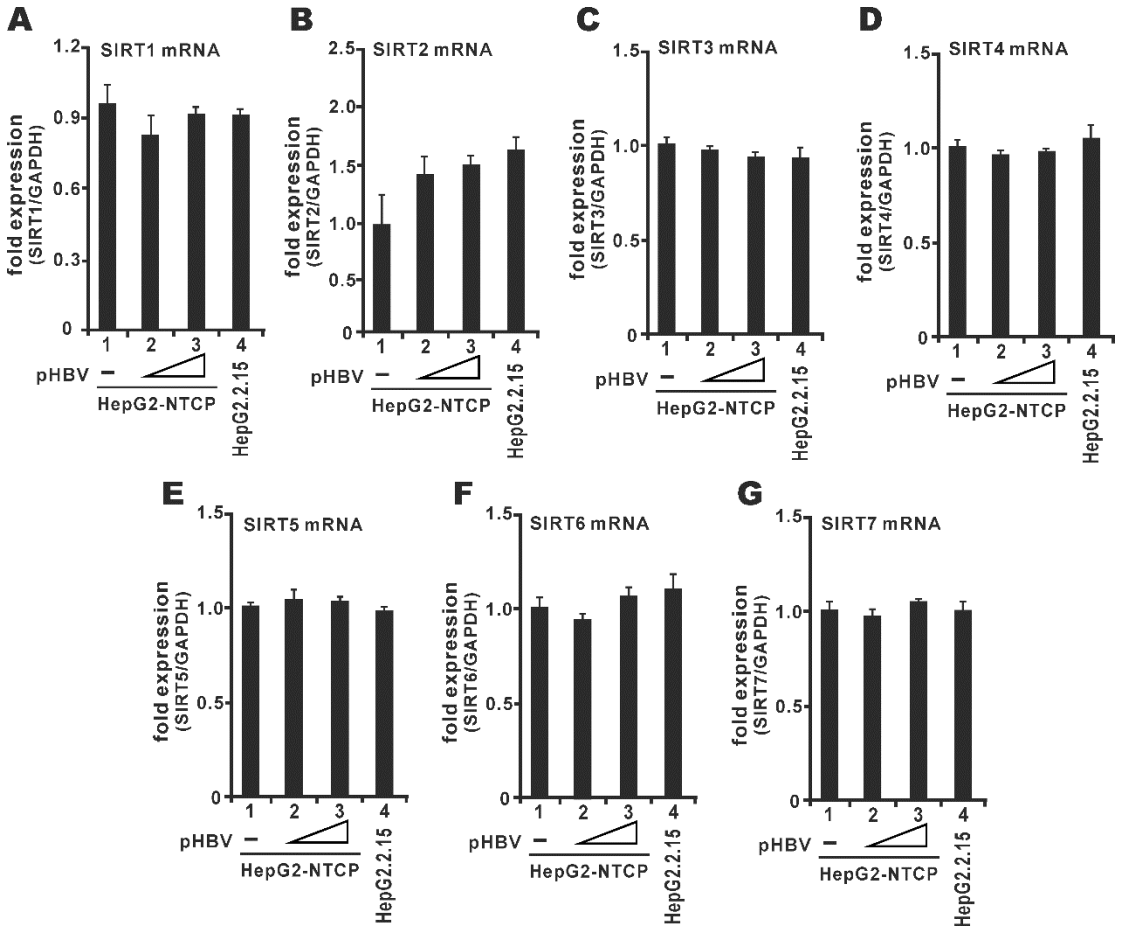

Fig. S2. mRNA levels of all sirtuins (SIRT1 to SIRT7) in pHBV1.3D-transfected HepG2-NTCP cells and in HepG2.2.15 cells. Cells were harvested 48 h post-transfection for RT-qPCR analysis.

Interplay between SIRT1 and hepatitis B virus X protein in the activation of viral transcription

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Highlights:

- All sirtuins activate HBV transcription.
- SIRT1 interacts with HBx and potentiates HBx activity.
- SIRT1 and HBx are mutually dependent in the activation of HBV transcription.
- SIRT1 inhibitors have anti-HBV activity.

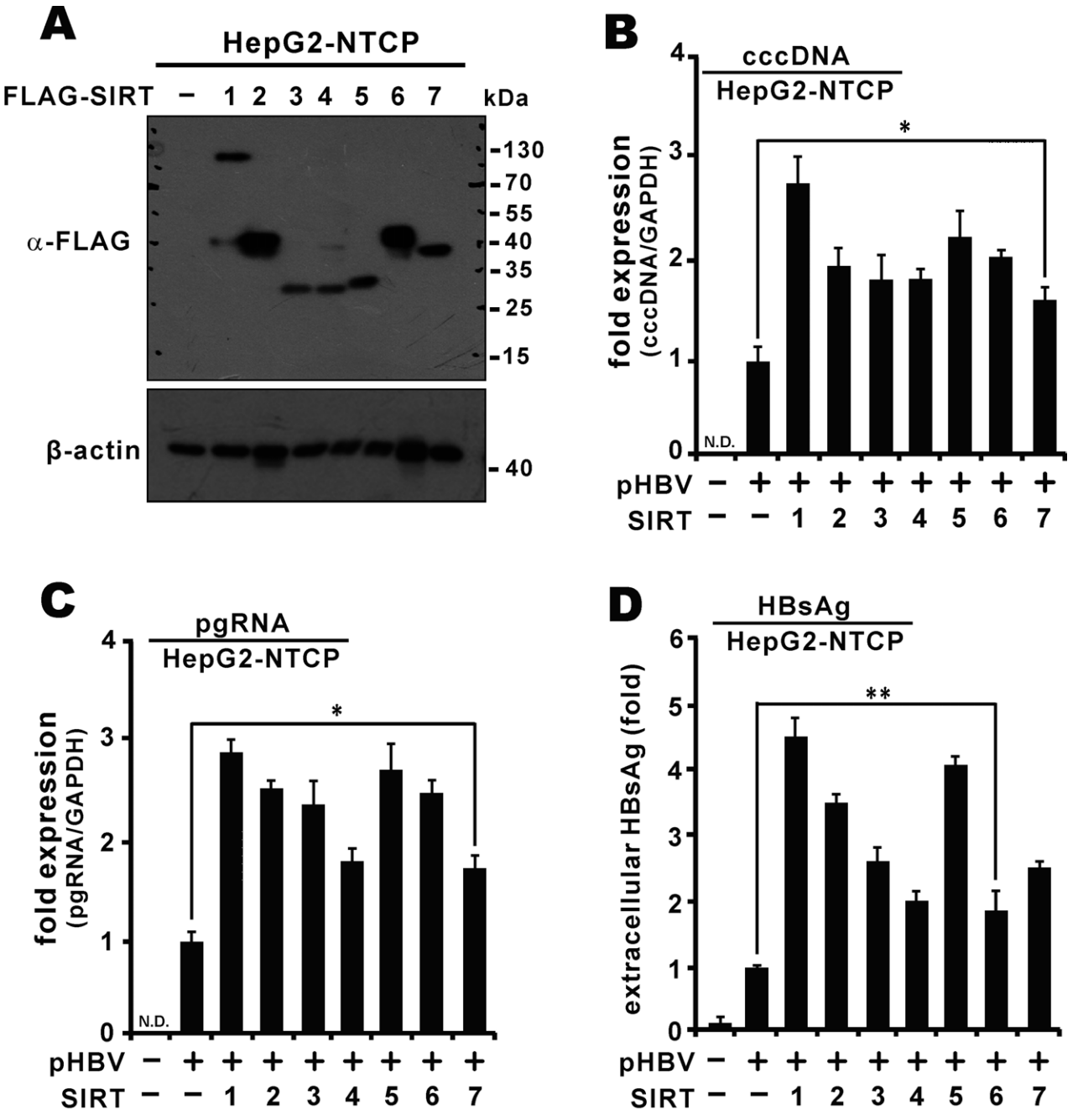

A
HepG2-NTCP
FLAG-SIRT – 1 2 3 4 5 6 7 kDa
α-FLAG
β-actin
130
70
55
40
35
25
15
40
B
cccDNA
HepG2-NTCP
fold expression
(cccDNA/GAPDH)
N.D.
*
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7
C
pgRNA
HepG2-NTCP
fold expression
(pgRNA/GAPDH)
N.D.
*
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7
D
HBsAg
HepG2-NTCP
extracellular HBsAg (fold)
**
pHBV – + + + + + + + +
SIRT – – 1 2 3 4 5 6 7

Figure 1

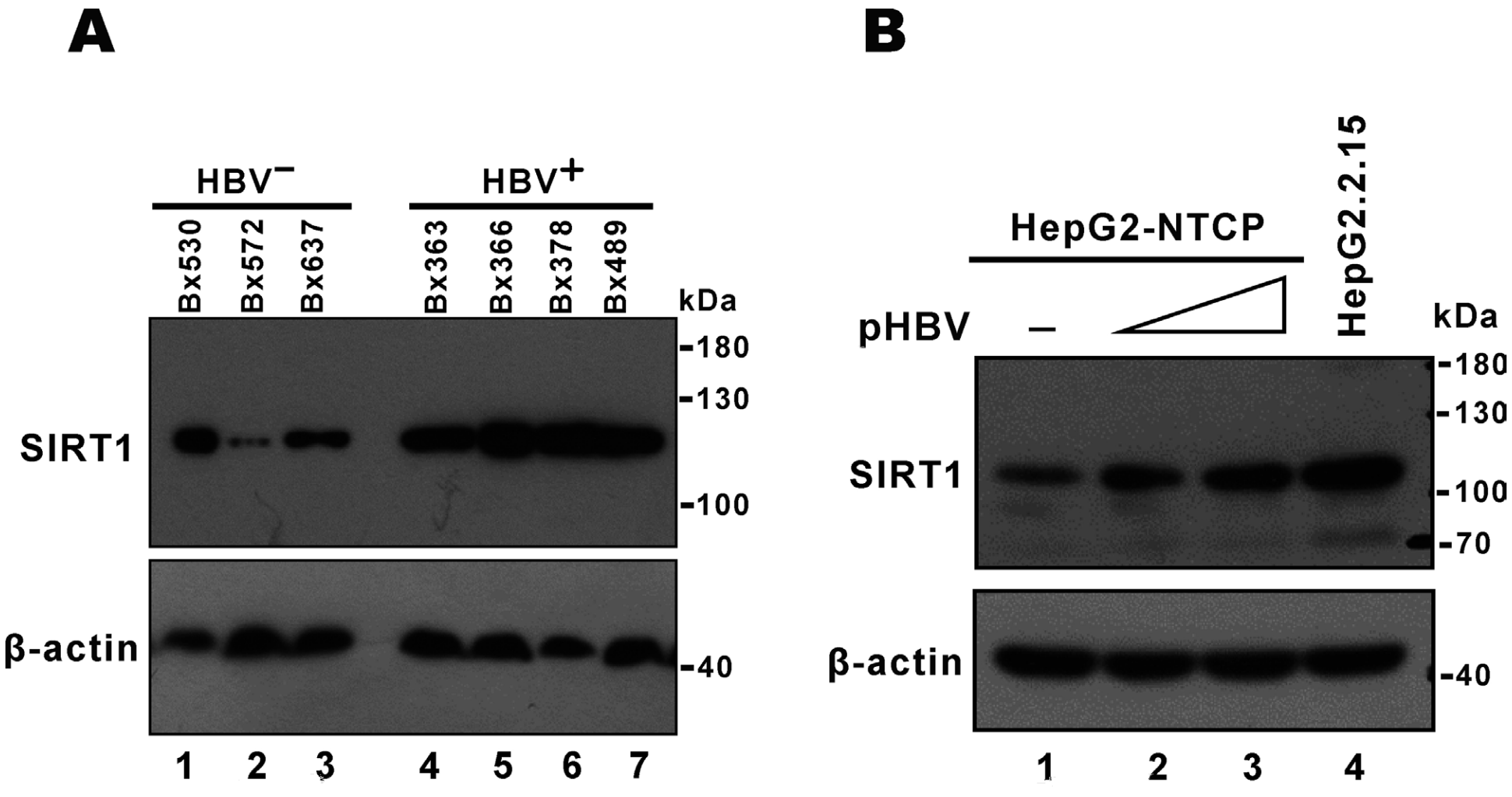

A
HBV−
HBV+
Bx530
Bx572
Bx637
Bx363
Bx366
Bx378
Bx489
kDa
SIRT1
β-actin
180
130
100
40
1 2 3 4 5 6 7
B
HepG2-NTCP
HepG2.2.15
pHBV
–
kDa
SIRT1
β-actin
180
130
100
70
40
1 2 3 4

Figure 2

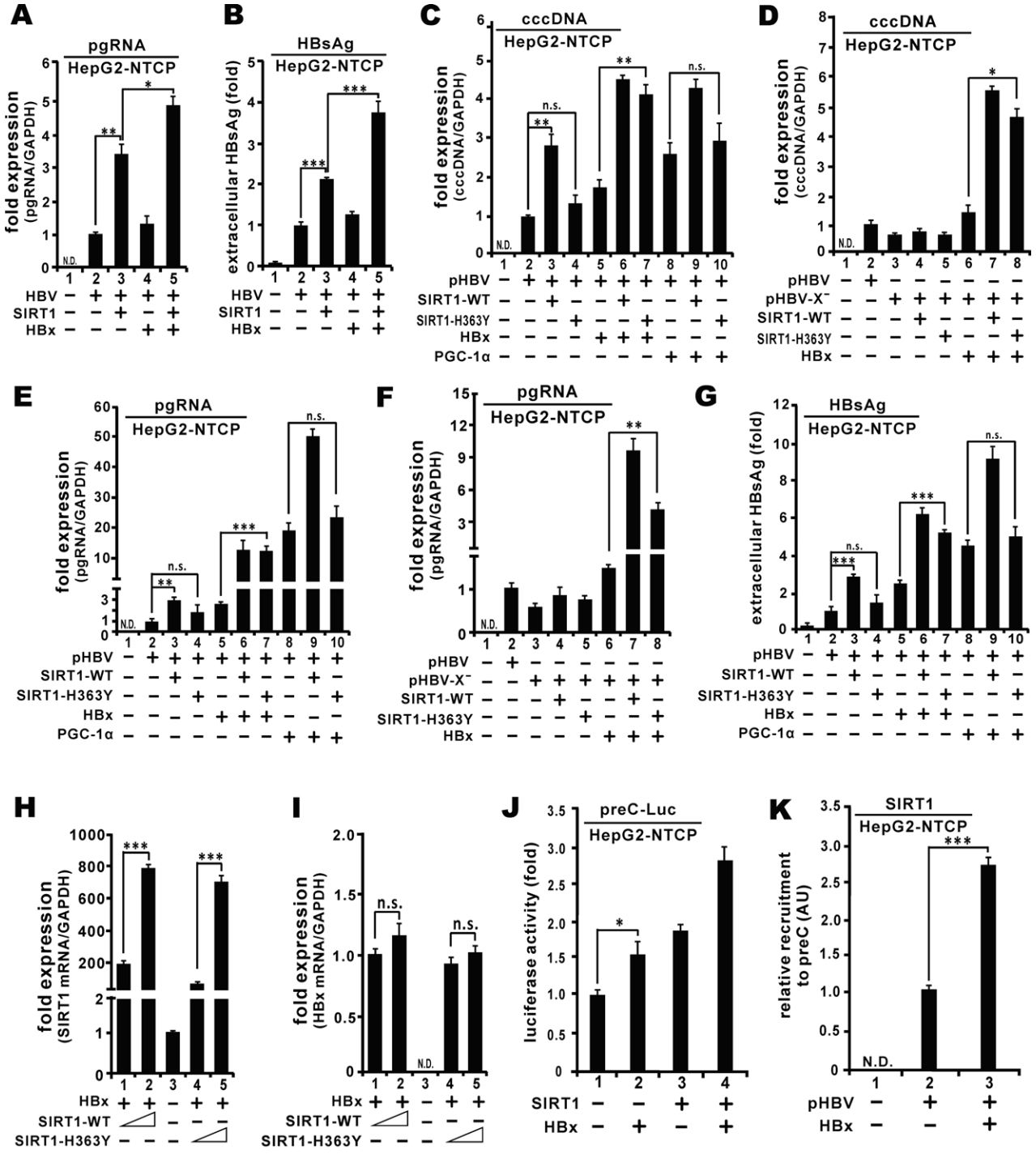

A
pgRNA
HepG2-NTCP
fold expression
(pgRNA/GAPDH)
N.D.
HBV
SIRT1
HBx
B
HBsAg
HepG2-NTCP
extracellular HBsAg (fold)
C
cccDNA
HepG2-NTCP
fold expression
(cccDNA/GAPDH)
n.s.
pHBV
SIRT1-WT
SIRT1-H363Y
HBx
PGC-1α
D
cccDNA
HepG2-NTCP
pHBV-X⁻
E
pgRNA
HepG2-NTCP
F
pgRNA
HepG2-NTCP
G
HBsAg
HepG2-NTCP
H
fold expression
(SIRT1 mRNA/GAPDH)
I
fold expression
(HBx mRNA/GAPDH)
J
preC-Luc
HepG2-NTCP
luciferase activity (fold)
K
SIRT1
HepG2-NTCP
relative recruitment
to preC (AU)

Figure 3

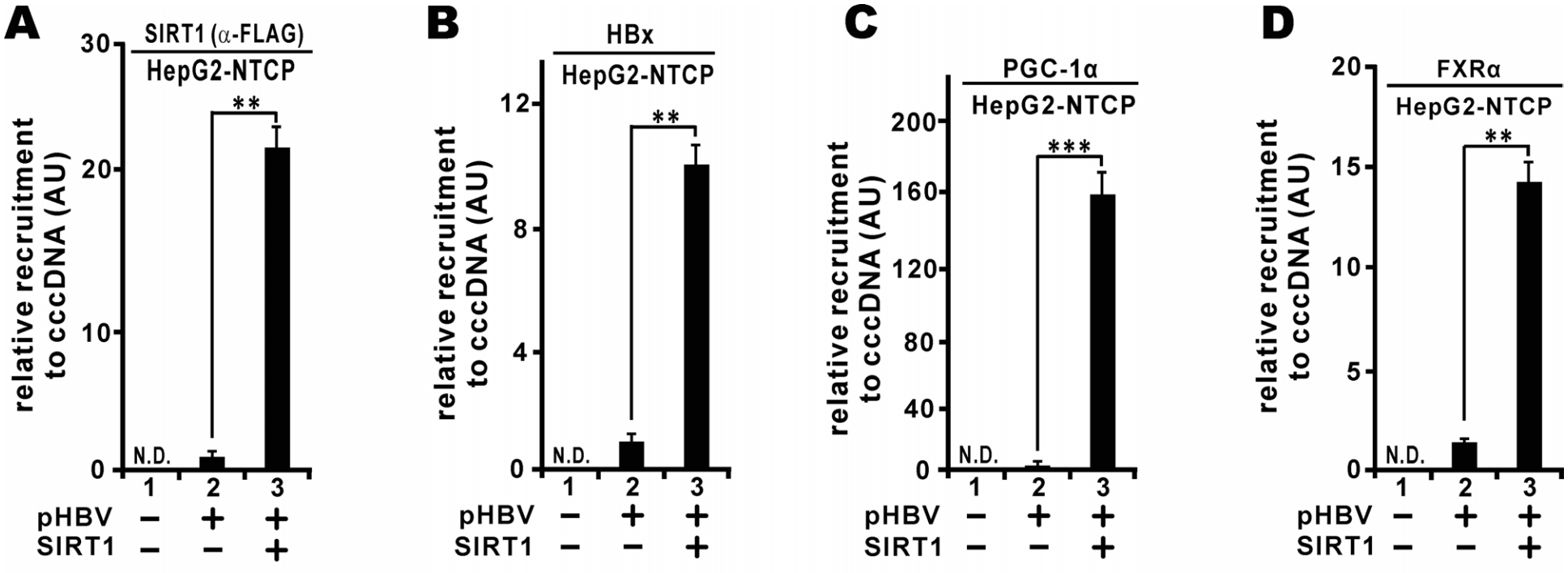

A
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to cccDNA (AU)
30
20
10
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
B
HBx
HepG2-NTCP
relative recruitment to cccDNA (AU)
12
8
4
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
C
PGC-1α
HepG2-NTCP
relative recruitment to cccDNA (AU)
200
160
120
80
40
0
***
N.D.
1
2
3
pHBV – + +
SIRT1 – – +
D
FXRα
HepG2-NTCP
relative recruitment to cccDNA (AU)
20
15
10
5
0
**
N.D.
1
2
3
pHBV – + +
SIRT1 – – +

Figure 4

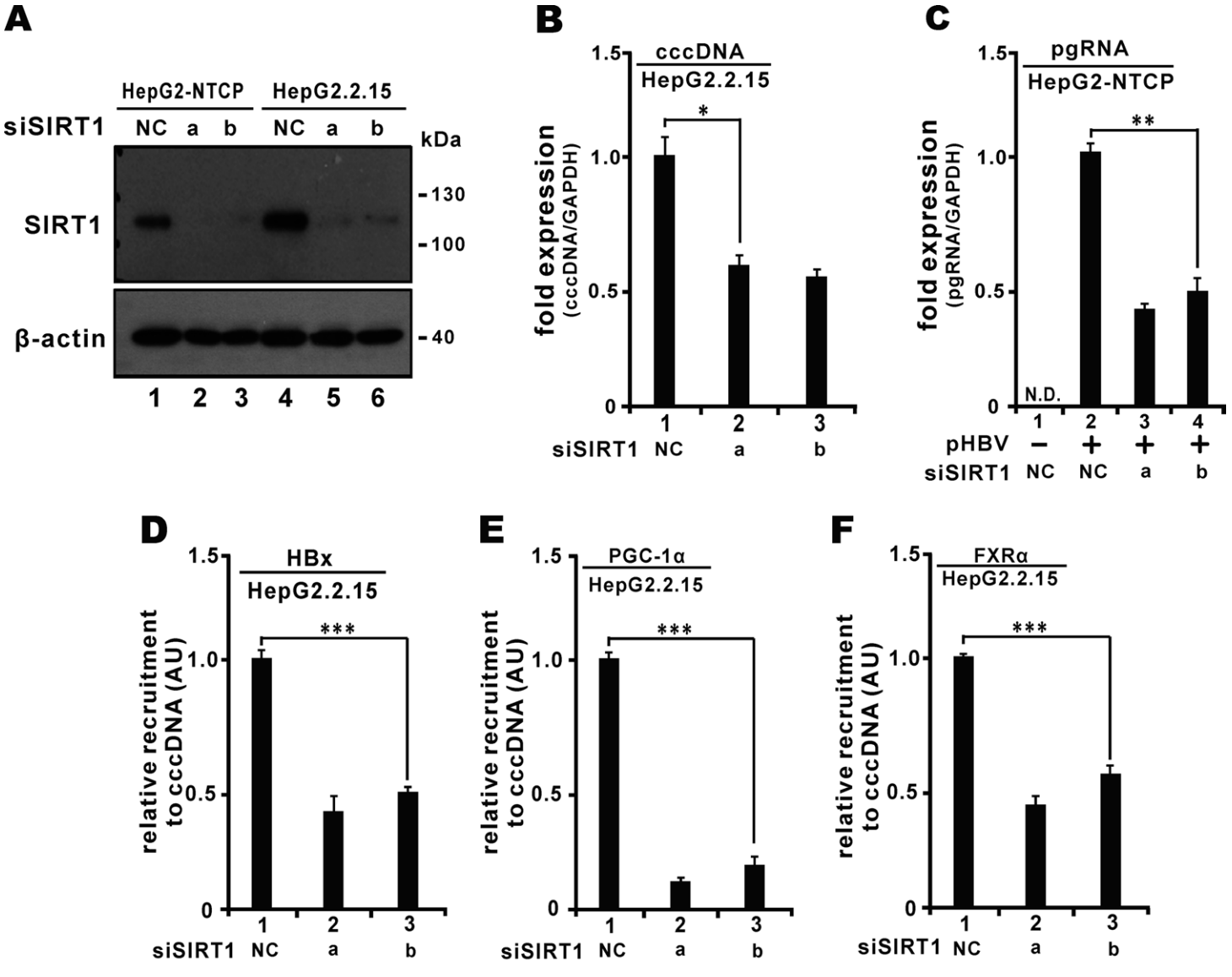

A
HepG2-NTCP
HepG2.2.15
siSIRT1
NC a b NC a b
kDa
SIRT1
β-actin
130
100
40
1 2 3 4 5 6
B
cccDNA
HepG2.2.15
fold expression
(cccDNA/GAPDH)
1.5
1.0
0.5
0
*
1 2 3
siSIRT1 NC a b
C
pgRNA
HepG2-NTCP
fold expression
(pgRNA/GAPDH)
N.D.
**
1 2 3 4
pHBV – + + +
siSIRT1 NC NC a b
D
HBx
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b
E
PGC-1α
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b
F
FXRα
HepG2.2.15
relative recruitment
to cccDNA (AU)
***
siSIRT1 NC a b

Figure 5

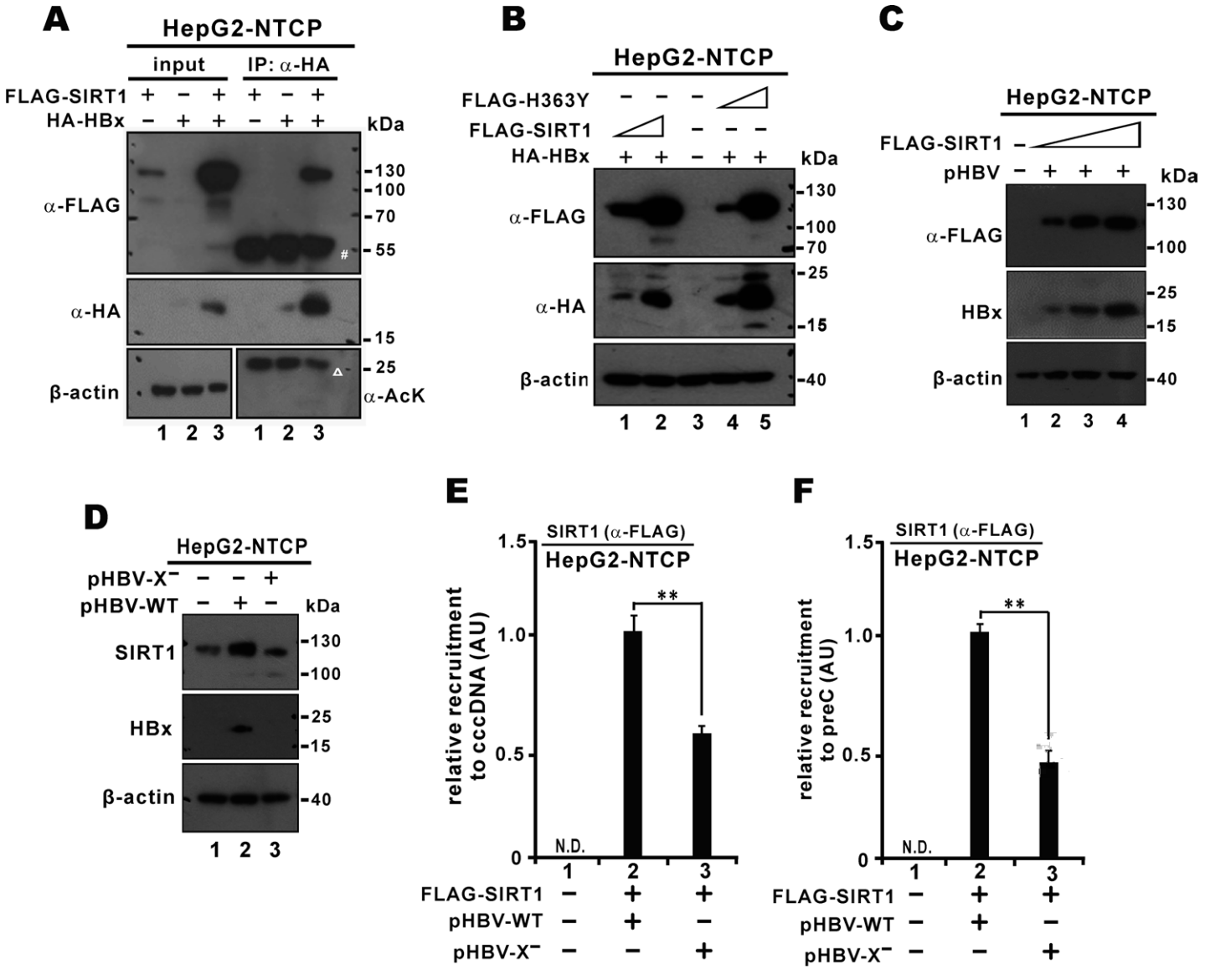

A
HepG2-NTCP
input
IP: α-HA
FLAG-SIRT1 + − + + − +
HA-HBx − + + − + +
kDa
α-FLAG
α-HA
β-actin
130
100
70
55
15
25
α-AcK
1 2 3 1 2 3
B
HepG2-NTCP
FLAG-H363Y
FLAG-SIRT1
HA-HBx + + − + +
kDa
α-FLAG
α-HA
β-actin
130
100
70
25
15
40
1 2 3 4 5
C
HepG2-NTCP
FLAG-SIRT1
pHBV − + + +
kDa
α-FLAG
HBx
β-actin
130
100
25
15
40
1 2 3 4
D
HepG2-NTCP
pHBV-X⁻ − − +
pHBV-WT − + −
kDa
SIRT1
HBx
β-actin
130
100
25
15
40
1 2 3
E
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to cccDNA (AU)
1.5
1.0
0.5
0
**
N.D.
1 2 3
FLAG-SIRT1 − + +
pHBV-WT − + −
pHBV-X⁻ − − +
F
SIRT1 (α-FLAG)
HepG2-NTCP
relative recruitment to preC (AU)
1.5
1.0
0.5
0
**
N.D.
1 2 3
FLAG-SIRT1 − + +
pHBV-WT − + −
pHBV-X⁻ − − +

Figure 6

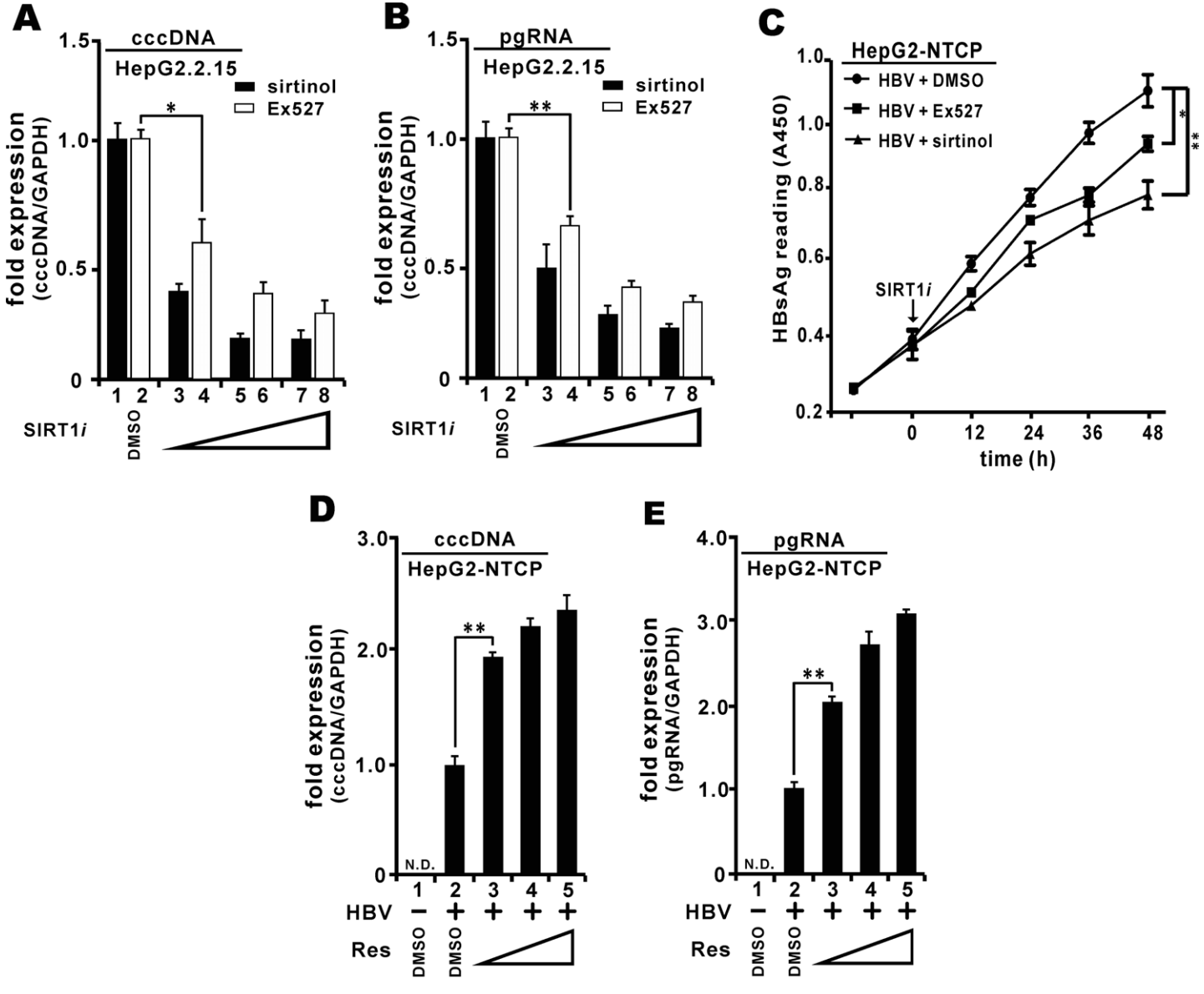

A
cccDNA
HepG2.2.15
sirtinol
Ex527
fold expression
(cccDNA/GAPDH)
SIRT1i
DMSO
B
pgRNA
HepG2.2.15
C
HepG2-NTCP
HBV + DMSO
HBV + Ex527
HBV + sirtinol
HBsAg reading (A450)
time (h)
D
cccDNA
HepG2-NTCP
N.D.
HBV
Res
E
pgRNA
HepG2-NTCP
(pgRNA/GAPDH)

Figure 7

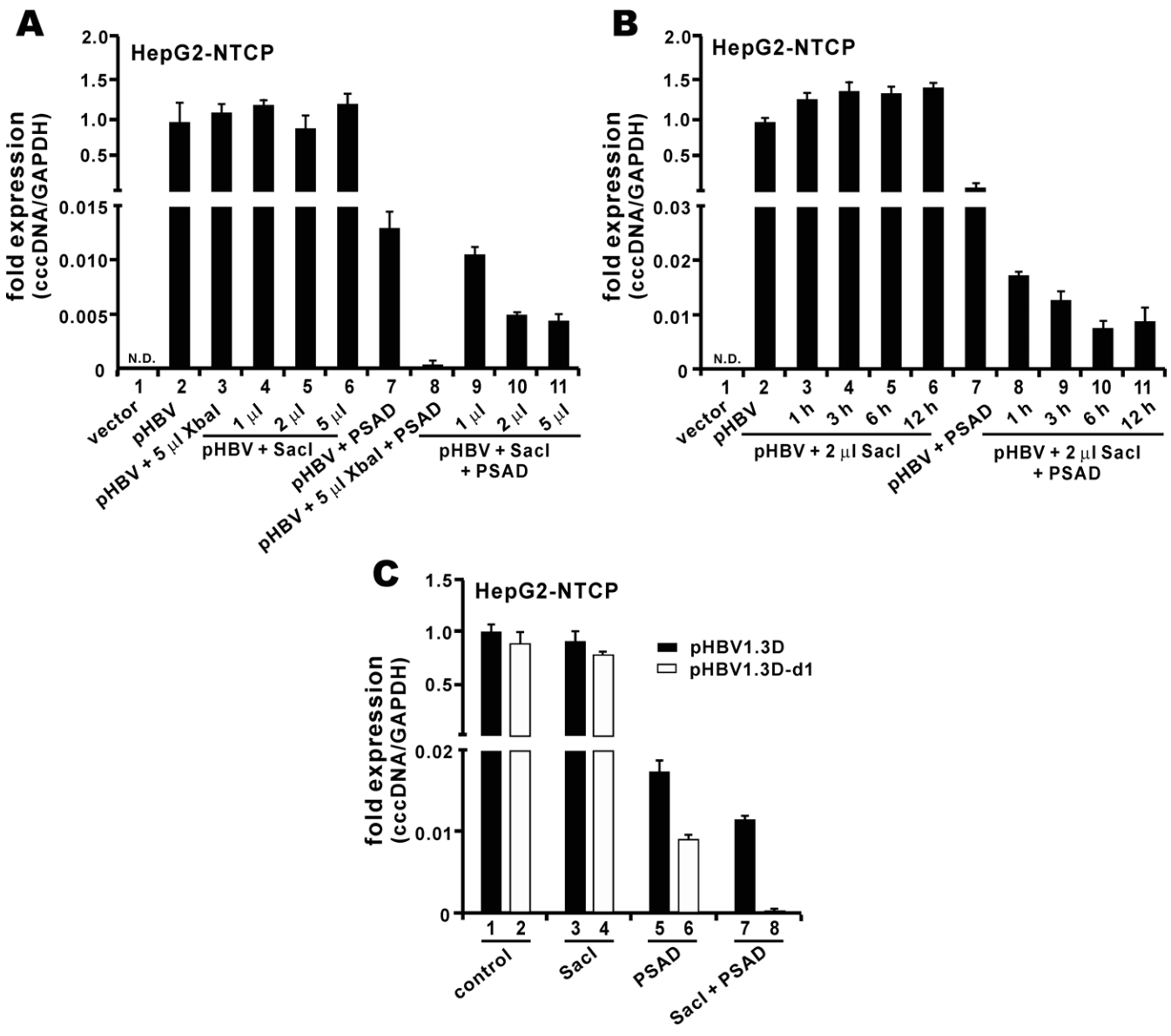

A
HepG2-NTCP
fold expression (cccDNA/GAPDH)
2.0
1.5
1.0
0.5
0.015
0.010
0.005
0
N.D.
1 2 3 4 5 6 7 8 9 10 11
vector
pHBV
pHBV + 5 μl XbaI
1 μl
2 μl
5 μl
pHBV + SacI
pHBV + PSAD
pHBV + 5 μl XbaI + PSAD
1 μl
2 μl
5 μl
pHBV + SacI + PSAD
B
HepG2-NTCP
fold expression (cccDNA/GAPDH)
2.0
1.5
1.0
0.5
0.03
0.02
0.01
0
N.D.
1 2 3 4 5 6 7 8 9 10 11
vector
pHBV
1 h
3 h
6 h
12 h
pHBV + 2 μl SacI
pHBV + PSAD
1 h
3 h
6 h
12 h
pHBV + 2 μl SacI + PSAD
C
HepG2-NTCP
fold expression (cccDNA/GAPDH)
1.5
1.0
0.5
0.02
0.01
0
pHBV1.3D
pHBV1.3D-d1
1 2 3 4 5 6 7 8
control
SacI
PSAD
SacI + PSAD

Figure S1

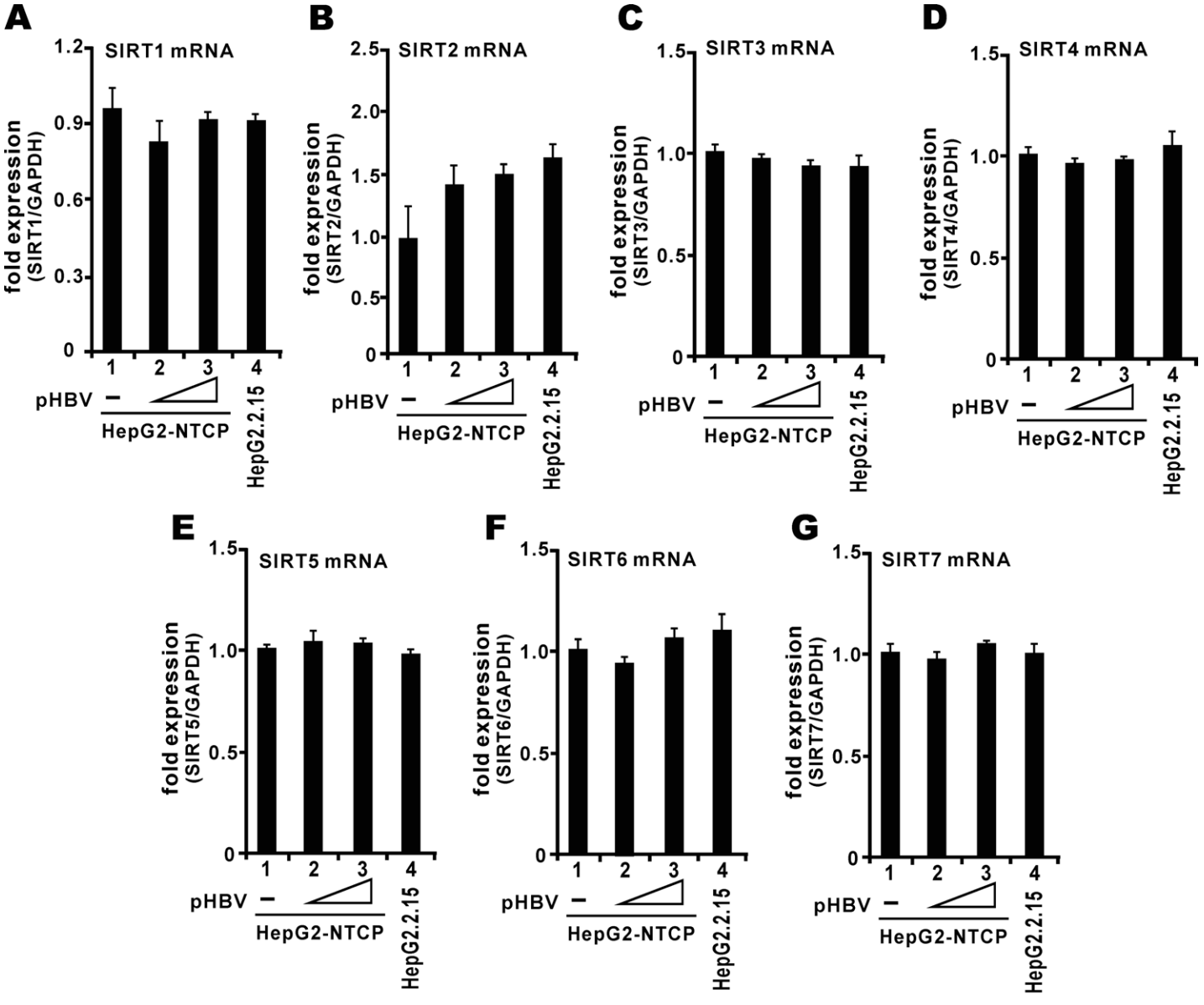
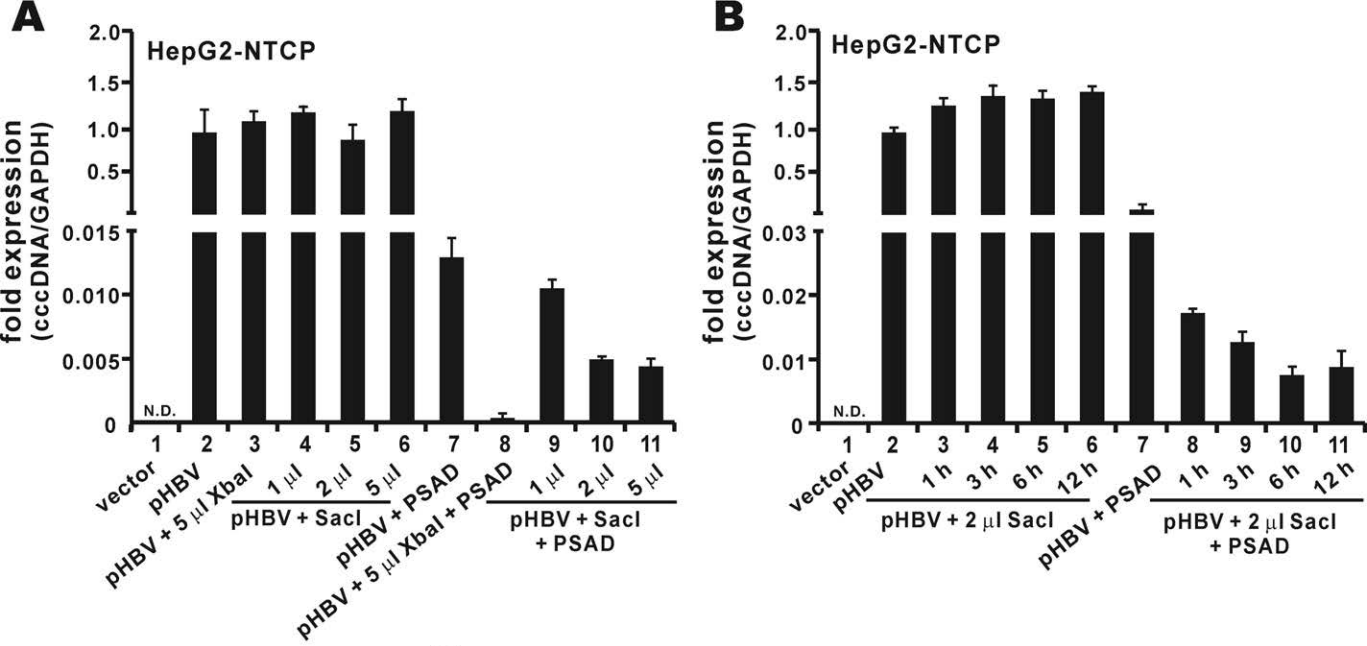

A
SIRT1 mRNA
fold expression (SIRT1/GAPDH)
1.2
0.9
0.6
0.3
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
B
SIRT2 mRNA
fold expression (SIRT2/GAPDH)
2.5
2.0
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
C
SIRT3 mRNA
fold expression (SIRT3/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
D
SIRT4 mRNA
fold expression (SIRT4/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
E
SIRT5 mRNA
fold expression (SIRT5/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
F
SIRT6 mRNA
fold expression (SIRT6/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15
G
SIRT7 mRNA
fold expression (SIRT7/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – ▲
HepG2-NTCP
HepG2.2.15

Figure S2


A
HepG2-NTCP
fold expression (cccDNA/GAPDH)
2.0
1.5
1.0
0.5
0.015
0.010
0.005
0
N.D.
1 2 3 4 5 6 7 8 9 10 11
vector
pHBV
pHBV + 5 μl XbaI
1 μl
2 μl
5 μl
pHBV + SacI
pHBV + PSAD
pHBV + 5 μl XbaI + PSAD
1 μl
2 μl
5 μl
pHBV + SacI + PSAD
B
HepG2-NTCP
fold expression (cccDNA/GAPDH)
2.0
1.5
1.0
0.5
0.03
0.02
0.01
0
N.D.
1 2 3 4 5 6 7 8 9 10 11
vector
pHBV
1 h
3 h
6 h
12 h
pHBV + 2 μl SacI
pHBV + PSAD
1 h
3 h
6 h
12 h
pHBV + 2 μl SacI + PSAD

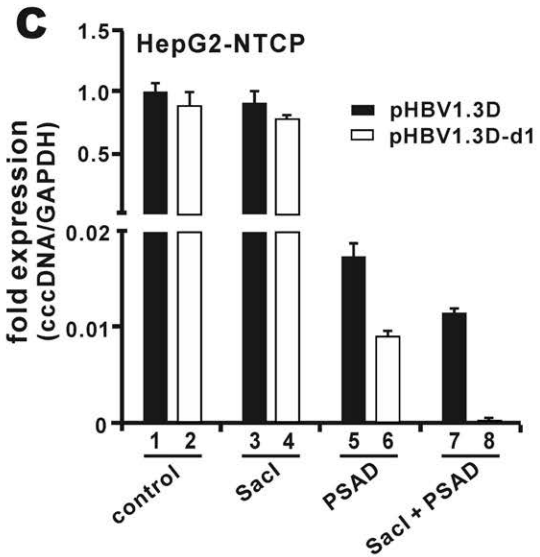

C
HepG2-NTCP
fold expression (cccDNA/GAPDH)
1.5
1.0
0.5
0.02
0.01
0
pHBV1.3D
pHBV1.3D-d1
1 2 3 4 5 6 7 8
control
SacI
PSAD
SacI + PSAD

Figure S1

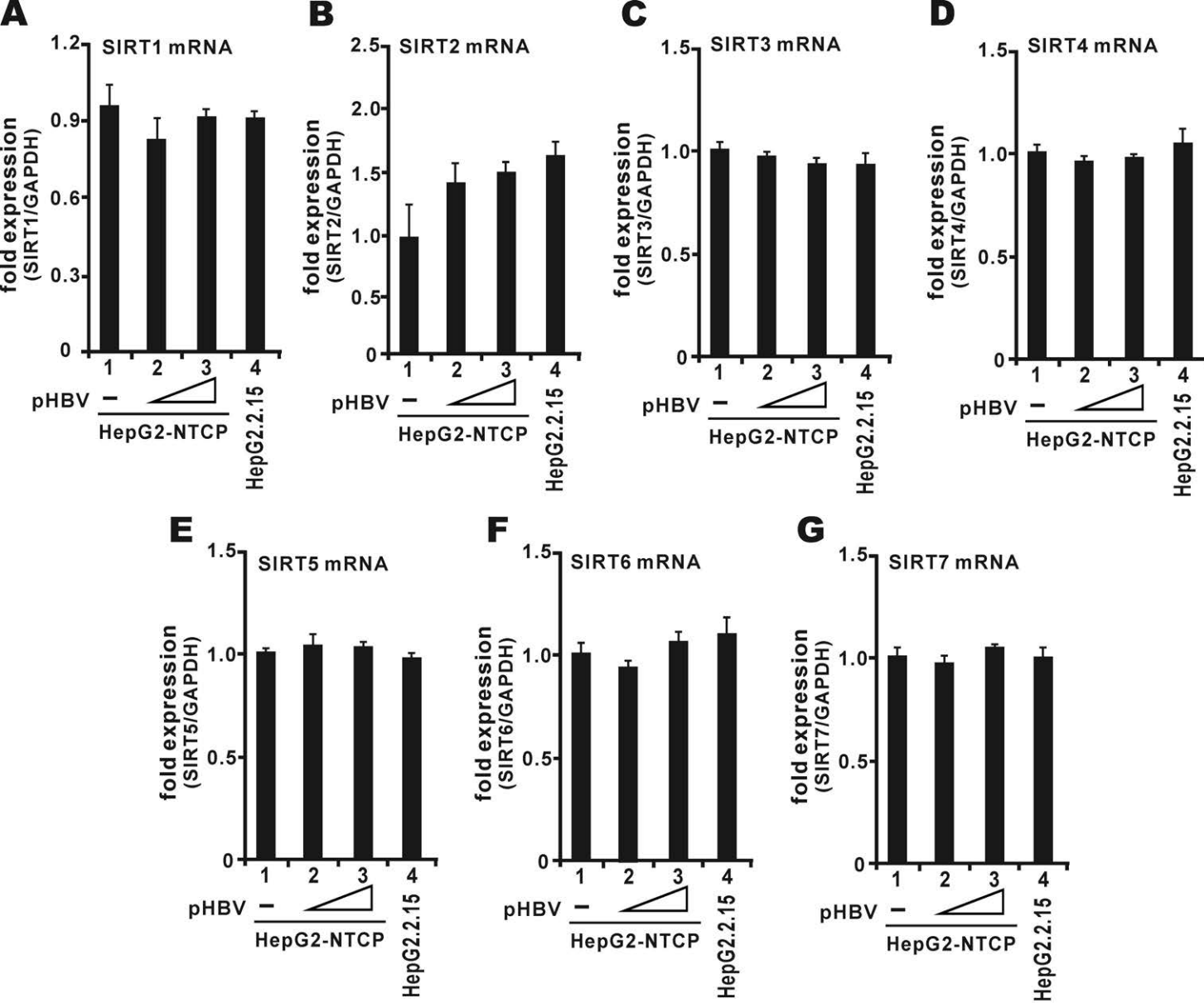

A
SIRT1 mRNA
fold expression (SIRT1/GAPDH)
1.2
0.9
0.6
0.3
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
B
SIRT2 mRNA
fold expression (SIRT2/GAPDH)
2.5
2.0
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
C
SIRT3 mRNA
fold expression (SIRT3/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
D
SIRT4 mRNA
fold expression (SIRT4/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
E
SIRT5 mRNA
fold expression (SIRT5/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
F
SIRT6 mRNA
fold expression (SIRT6/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15
G
SIRT7 mRNA
fold expression (SIRT7/GAPDH)
1.5
1.0
0.5
0
1 2 3 4
pHBV – +
HepG2-NTCP
HepG2.2.15

Figure S2