Forkhead O transcription factor 4 restricts HBV covalently closed circular DNA transcription and HBV replication through genetic downregulation of hepatocyte nuclear factor 4 alpha and epigenetic suppression of covalently closed circular DNA via interacting with promyelocytic leukemia protein

Yuqi Li1#, Minjing He1#, Ruijie Gong1#, Ziteng Wang1, Lin Lu1, Shu Peng1, Zhiyun Duan1, Ying Feng1, Yi Liu3* and Bo Gao1,2*

#These authors contribute equally to this work.

1From the Department of Immunology, School of Basic Medical Sciences, Shanghai Medical College of Fudan University, Shanghai 200032, P.R. China

2Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, P.R. China

3Department of Digestive Diseases, Huashan Hospital, Fudan University, Shanghai 200040, P.R. China

*Corresponding authors: Bo Gao, E-mail: gaobofd@126.com; or Yi Liu, E-mail: hsluyi0205@163.com
Supplemental Figures and Tables

Figure S1

**Figure S1.** FoxO4 inhibits rcccDNA-mediated transcription and HBV replication in mice. Mice were injected intravenously with prcccDNA/Cre and FoxO4-Flag or vector using HGT technique (n = 5). 4 days post-injection, mice liver tissues and sera
were collected. (A) The protein level of FoxO4-Flag in mice liver tissues was determined by Western blotting using antibodies against Flag and GAPDH. (B, C) The levels of HBsAg and HBV DNA in mice sera were determined by ELISA and qPCR, respectively. (D) The level of HBeAg in the liver tissues of mice was analyzed by immunohistochemical staining. (E, F) The levels of HBV RNAs and preC-pgRNA in the liver tissues of mice were analyzed by qRT-PCR. (G) The level of HBV DNA in the liver tissues of mice was determined by Southern blotting. Data are shown as means ± SD and are representative of three independent experiments. Scale bar: 100 μm. ****P < 0.0001. NS, no significance; RI, replicative intermediates.

Supporting FIG. S2

Supporting FIG. S2. FoxO4 displays epigenetic suppressive activity on rcccDNA in vivo. Mice were injected with prcccDNA/Cre and FoxO4-Flag or vector using HGT technique (n = 5). 4 days post-injection, mice liver tissues were collected. The effect of FoxO4 on the recruitment of AcH3, H3K4me3, H3K9me3, and H3K27me3 onto rcccDNA in mice liver tissues was determined by ChIP assays. Data are shown as fold change to control empty vector-transfected cells after normalized to input and control IgG. The value obtained from control siRNA-transfected and IRF-1-untreated mice was set to 1. Data are shown as means ± SD and are representative of three independent experiments. **P < 0.01.
Supporting FIG. S3. The role of IFI16 in FoxO4-mediated anti-cccDNA activity. A, B: FoxO4-Flag was transfected into Huh7 cells for 48 h, and the protein level of exogenous FoxO4-Flag was determined by Western blotting (A), and the mRNA level of IFI16 was determined by qRT-PCR (B). C, D: Empty control vector, FoxO4-Flag or IFI16-Flag was transfected into Huh7 cells together with pre-cccDNA/Cre for different time points (24, 48, 72 hours). The exogenously-expressed FoxO4-Flag and IFI16-Flag were detected by Western blotting (C), and the mRNA levels of IFN-β, OAS1 and ISG56 (D) were examined by qRT-PCR for the indicated time points. E, F: Huh7 cells were transfected with IFI16 siRNA for 48 h, and the cells were further transfected with FoxO4-Flag for another 48 h. The expression level of exogenous FoxO4 was determined by Western blotting (E), and the HBV proteins, HBV transcripts and HBV DNA was examined by ELISA, qRT-PCR and qPCR,
respectively (F). Data are shown as means ± SD and are representative of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. NS, no significance.

Supporting FIG. S4

(A) FoxO4-HA and p53-Flag were co-transfected into Huh7 cells. After 48 h transfection, immunoprecipitation was performed using mouse M2 anti-Flag or IgG, and immunoblotting was then performed using rabbit anti-Flag, rabbit anti-HA and GAPDH. (B) The protein level of p53 in HepG2, Huh7 and Hep3B cells was determined by Western blotting using antibodies against p53 and GAPDH. (C-F) The role of p53 in FoxO4-mediated suppression of rcccDNA-driven transcription and HBV replication in HepG2, Huh7 and Hep3B cells was determined by ELISA, qRT-PCR and qPCR, respectively. Data
are shown as means ± SD and are representative of three independent experiments. **P < 0.01. NS, no significance. The differences within and between groups were compared by Student’s t-test and two-way analysis of variance (ANOVA), respectively.

Supporting FIG. S5

Supporting FIG. S5. PML do not affect the expression of HNF4α in the absence or presence of FoxO4. Huh7 cells (A) or HepG2-NTCP cells (B) were transfected with control or PML siRNA. 48 hours later, cells were further transfected with FoxO4-Flag or control empty vector for another 48 hours. The expression levels of FoxO4-Flag, PML and HNF4α were determined by Western blotting.
Supporting FIG. S6. FoxO4 fails to upregulate the expression level of SMC5 and SMC6 and inhibits the rcccDNA-driven transcription and HBV replication independent of HBx. A: FoxO4-Flag or control empty vector was transfected into Huh7 cells together with rcccDNA/Cre plasmids. 24 hours posttransfection, the protein level of FoxO4, SMC5 and SMC6 were examined by Western blot. B, C: FoxO4-Flag was transfected into Huh7 cells together with rcccDNA/Cre (WT) or rcccDNA with the HBx mutated/Cre (HBx mutant). 48 hours posttransfection, levels of preC-pgRNA (B) and HBV DNA (C) were determined by qRT-PCR and qPCR, respectively. Data are shown as means ± SD of triplicates and are representative of three independent experiments. **P < 0.01. NS, no significance.
Supporting FIG. S7. Graphical illustration of FoxO4-mediated inhibitory effect on HBV cccDNA. FoxO4 displays inhibitory effect on cccDNA-driven transcription and HBV replication, but it does not affect the level of cccDNA itself. Mechanistically, FoxO4 expression could lead to the epigenetic suppression of cccDNA through co-localizing with PML in the nuclear bodies and interacting with PML. Downregulation of PML significantly attenuates FoxO4-mediated epigenetic suppression of cccDNA and the following cccDNA transcription and HBV production. On the other hand, FoxO4 expression leads to the downregulation of HNF4α. However, HNF4α appears not to be involved in FoxO4-mediated epigenetic suppression of cccDNA, although it contributes indeed to FoxO4 inhibition of HBV core promoter activity. Together, FoxO4 might inhibit cccDNA transcription and HBV replication via a two-part mechanism: one is epigenetic suppression of cccDNA via interacting with PML, the other is inhibition of HBV core promoter activity involving the genetic downregulation of HNF4α.
**Supplemental Table 1: Primers/siRNA oligos**

| Category          | Forward             | Reverse                   |
|-------------------|---------------------|---------------------------|
| **HBV RNAs**      |                     |                           |
|                   | 5'-GCACCTCGCTTCACCTCTGC-3' | 5'-CTCAAGGTCGTCGTTGACA-3' |
| **preC-pgRNA**    | 5'-TGTTCAAGCCTCAAGGCT-3'    | 5'-GGAAAGAACGTACAGGCAA-3' |
| **HBV DNA**       | 5'-CCCCTTTGGTCTCTCATTCC-3' | 5'-GTCCGAAGGTTGGTACAGC-3' |
| **HBV cccDNA**    | 5'-CTCCCCGTCTGCTGCTTCT-3'    | 5'-GCCCAAGCCACCCCAAG-3'   |
| **HBV rcccDNA**   | 5'-CAAGCACAGGTGTTAAGGAGAC-3'     | 5'-GAGAGAAAGGCAAAGTGGAT-3' |
| **β-globin**      | 5'-GTGCACCTGACTCTGAGGAGA-3'     | 5'-CCTTGATACCAACTGCCCCAG-3' |
| **GAPDH**         | 5'-GCCTCTGCGCCCTAGCTA-3'      | 5'-GATGCGGCGGTCTCTGGAAC-3' |
| **IFN-β**         | 5'-GACCAACAAGTGTCTCTCCTCAA-3'     | 5'-AACTGCTGCAGCTGCTTAATC-3' |
| **OAS1**          | 5'-TCCACCTGCTTCACAGAAGTACA-3'     | 5'-TGCGGCTGTGTGAAAATGTGGTT-3' |
| **ISG56**         | 5'-GCCTTGCTGAGTGTTGAGGAAA-3'     | 5'-ATCCAGGCGAAGGAGGAT-3'   |
| **FoxO4 siRNA#1** | Sense 5'-CGCGAUCAUAGACCUAGAUU-3' | Anti-sense 5'-AUCUAGGAUGAUUGUACC-3' |
| **FoxO4 siRNA#2** | Sense 5'-GUGACAUAGGAUACUAUAGATT-3' | Anti-sense 5'-AUGAUGUUAUCCAUGCACTT-3' |
| **PML siRNA**     | Sense 5'-AGAUGUCACGUGUAAUCCAG-3' | Anti-sense 5'-CUUGGAUACAGCUGCAUCU-3' |
## Supplemental Table 2: Antibodies

| Antibody   | Source                                      |
|------------|---------------------------------------------|
| Anti-Flag  | Bioworld Biotechnology, AP0007              |
| GAPDH      | Cell Signaling Technology, #8884            |
| FoxO4      | Cell Signaling Technology, #9472            |
| HBcAg      | Abcam, ab8639                               |
| H3         | Santa Cruz, sc-8654                        |
| AcH3       | Cell Signaling Technology, #9677            |
| H3K4me3    | Cell Signaling Technology, #9727            |
| H3K9me3    | Cell Signaling Technology, #5327            |
| H3K27me3   | Cell Signaling Technology, #9733            |
| Anti-HA    | Bioworld Biotechnology, AP0005              |
| PML        | Cell Signaling Technology, #33156           |
| p53        | Cell Signaling Technology, #2524            |
| HNF4α      | Bioworld Biotechnology, BS6888              |
| SMC5       | Abcam, ab154103                            |
| SMC6       | Abcam, ab155495                            |
## Supplemental Table S3 Characteristics of CHB patients and Control individuals

| No. | Age (yr) | Gender | HBsAg | Serum HBV-DNA (IU/mL) | Serum AST (IU/L) | Serum ALT (IU/L) | No. | Age (yr) | Gender | HBsAg | Serum HBV-DNA (IU/mL) | Serum AST (IU/L) | Serum ALT (IU/L) |
|-----|----------|--------|-------|----------------------|-----------------|-----------------|-----|----------|--------|-------|----------------------|-----------------|-----------------|
| CHB patients | | | | | | | Control individuals | | | | | | | |
| 1   | 38       | M      | +     | 1.91E+05             | 46              | 49              | 1   | 38       | M      |       |                      |                 |                 |
| 2   | 21       | M      | +     | 3.30E+03             | 25              | 38              | 2   | 45       | F      |       |                      |                 |                 |
| 3   | 36       | M      | +     | 7.09E+07             | 60              | 87              | 3   | 51       | M      |       |                      |                 |                 |
| 4   | 46       | M      | +     | 1.69E+03             | 18              | 27              | 4   | 39       | M      |       |                      |                 |                 |
| 5   | 32       | F      | +     | 6.76E+05             | 48              | 103             | 5   | 52       | M      |       |                      |                 |                 |
| 6   | 58       | F      | +     | 4.18E+03             | 24              | 23              | 6   | 37       | M      |       |                      |                 |                 |
| 7   | 35       | M      | +     | 1.91E+04             | 39              | 108             | 7   | 46       | F      |       |                      |                 |                 |
| 8   | 23       | F      | +     | 3.68E+06             | 18              | 17              | 8   | 24       | F      |       |                      |                 |                 |
| 9   | 52       | M      | +     | 1.07E+05             | 30              | 60              | 9   | 61       | M      |       |                      |                 |                 |
| 10  | 39       | M      | +     | 9.87E+03             | 29              | 82              | 10  | 38       | M      |       |                      |                 |                 |
| 11  | 54       | M      | +     | 8.76E+02             | 23              | 23              | 11  | 31       | M      |       |                      |                 |                 |
| 12  | 53       | M      | +     | 1.01E+03             | 29              | 30              | 12  | 45       | F      |       |                      |                 |                 |
| 13  | 45       | F      | +     | 1.01E+03             | 18              | 22              | 13  | 27       | M      |       |                      |                 |                 |
| 14  | 37       | F      | +     | 5.56E+05             | 51              | 57              | 14  | 34       | M      |       |                      |                 |                 |
| 15  | 36       | M      | +     | 4.48E+01             | 60              | 87              | 15  | 37       | F      |       |                      |                 |                 |
| 16  | 44       | M      | +     | 9.09E+04             | 34              | 27              | 16  | 39       | M      |       |                      |                 |                 |
| 17  | 37       | F      | +     | 6.68E+05             | 31              | 34              | 17  | 41       | F      |       |                      |                 |                 |
| 18  | 39       | M      | +     | 5.68E+08             | 42              | 48              | 18  | 29       | M      |       |                      |                 |                 |