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## Hepatitis B Virus X protein promotes degradation of SMC5/6 to enhance HBV replication

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### SUMMARY

The Hepatitis B Virus (HBV) regulatory protein X (HBx) activates gene expression from the HBV covalently closed circular (cccDNA) genome. Interaction of HBx with the DDB1-CUL4-ROC1 (CRL4) E3 ligase is critical for this function. Using substrate-trapping proteomics, we identified the structural maintenance of chromosomes (SMC) complex proteins SMC5/6 as CRL4<sup>HBx</sup> substrates. HBx expression and HBV infection degraded the SMC5/6 complex in human hepatocytes in vitro and in humanized mice in vivo. HBx targets SMC5/6 for ubiquitylation by the CRL4<sup>HBx</sup> E3 ligase and subsequent degradation by the proteasome. Using a minicircle HBV (mcHBV) reporter system with HBx-dependent activity, we demonstrate that SMC5/6 knockdown, or inhibition with a dominant-negative SMC6, enhance HBx-null mcHBV-Gluc gene expression. Furthermore, SMC5/6 knockdown rescued HBx-deficient HBV replication in human hepatocytes. These results indicate that a primary function of HBx is to degrade SMC5/6, which restricts HBV replication by inhibiting HBV gene expression.

### Graphical Abstract

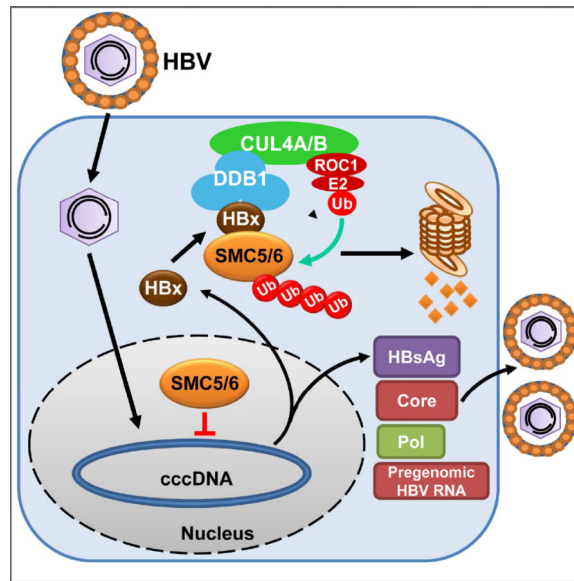
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#### Author Contributions

Contribution: C.M. planned, designed, and performed the experiments and wrote the paper; Y-P.X. performed SMC5/6 ubiquitination experiments; F.L. developed the HBV cccDNA reporter system, HBV infection in HepG2-NTCP and humanized mouse models; F.L. and K.N. performed NRG/FAH-hu liver mouse construction and HBV infection; C.M. and N.R.B. performed the co-IF imaging assays, XL and YW performed HBV infection experiments; X.Y. and L.S. conceived the research project, and wrote the paper.



## INTRODUCTION

Hepatitis B virus (HBV) infection causes chronic hepatitis B in an estimated 350 million people worldwide, putting these people at high risk for developing liver cirrhosis and eventually hepatocellular carcinoma (HCC) (Dienstag, 2008; Revill et al., 2016; Scaglione and Lok, 2012). HBV is a partially double-stranded DNA virus that belongs to the *Hepadnaviridae* family (Seeger et al., 2007). After entry into host cells, the viral genome is transported into the nucleus and converted to a covalently closed circular DNA (cccDNA), which is the transcription template for all HBV viral RNAs. Currently available HBV therapeutics, including interferon- $\alpha$  and antiviral drugs, fail to eradicate the cccDNA reservoir from infected hepatocytes, despite suppressing new viral DNA replication. Failure to repress or eliminate this cccDNA results in viral rebound after therapy (Nassal, 2015; Revill et al., 2016; Zeisel et al., 2015).

The HBV-encoded regulatory protein hepatitis B virus X protein (HBx) stimulates HBV gene expression from the cccDNA template, but the mechanism by which HBx facilitates HBV replication remains unclear (Keasler et al., 2007; Leupin et al., 2005; Slagle and Bouchard, 2016; Tang et al., 2005). HBx interacts with several cellular proteins and may mediate its role in virus replication through these interactions. The best-characterized HBx binding partner is the damage-specific DNA binding protein 1 (DDB1) (Lee et al., 1995; Sitterlin et al., 1997). The interaction between HBx and DDB1 is conserved among the HBx proteins from all mammalian *hepadnaviruses* and woodchuck hepatitis virus (WHV) X protein (Sitterlin et al., 1997). This binding is essential for HBV replication (Hodgson et al., 2012; Leupin et al., 2005). HBx has been shown to enhance HBV gene expression from episomal cccDNA. However, the mechanism and functional significance of HBx-DDB1 interaction during infection remains elusive.

Initially discovered as a DNA repair factor, DDB1 is now recognized to mainly function as a linker protein for the assembly of a large number of Cullin 4-ROC1/RING E3 ubiquitin ligase (CRL4) complexes (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). DDB1 bridges CUL4 to individual DDB1-binding WD40 proteins (DWD, or DDB Cullin Associated Factors, DCAFs), which in turn recruit substrates to the CUL4-ROC1 catalytic core for subsequent ubiquitination [reviewed in (Jackson and Xiong, 2009; Lee and Zhou, 2007)]. A structural study has revealed that HBx, although lacking a typical WD40 domain as found in other DWD/DCAF proteins, contains an  $\alpha$ -helical motif termed the H-box which is shared by several DWD proteins and some viral proteins and directly binds to DDB1 (Li et al., 2010). These findings suggest that HBx may assemble an HBx-DDB1-CUL4-ROC1 E3 ligase complex (referred to as CRL4<sup>HBx</sup> hereafter) to target host proteins that antagonize HBV replication for ubiquitylation and degradation. This study aims to identify substrates of the CRL4<sup>HBx</sup> E3 ligase that function as host restriction factors to inhibit HBV replication.

## RESULTS

### Identification of HBx substrates by substrate-trapping proteomics

To identify the substrate of CRL4<sup>HBx</sup>, we performed tandem affinity purification of HBx from a stable HepG2 cell line that inducibly expresses a biologically active HBx with N-terminal FLAG and SBP tags (Figure 1A and Figure S1A–C). Because HBx functions as part of an active E3 ligase, we reasoned that HBx binding to its substrate is transient and results in latter's degradation, preventing direct identification of the substrate through binding. We therefore treated cells with MLN4924, an inhibitor of Cullin-RING ligases that prevents enzymatic activity by preventing neddylation of the cullin subunit. Inhibition of CRL4 activity in this manner has been successfully used to identify CRL4 ubiquitylation substrates [e.g. (Emanuele et al., 2011; Tan et al., 2013)]. HBx expression was induced at low levels for 24 hours by the addition of doxycycline and then cells treated either with MLN4924 to stabilize HBx-substrate interactions, or DMSO as a control. HBx-interacting proteins were then purified by sequential anti-FLAG and streptavidin binding and tryptic peptides were analyzed by LC-MS/MS. This analysis, as expected, identified many components of the HBx-CRL4 complex, including DDB1, CUL4A, and CUL4B, and six subunits of COP9/Signalosome, indicating that HBx assembles into active CRL4 complexes. Since CUL4A and CUL4B are localized preferentially in the cytoplasm and the nucleus, respectively (Nakagawa and Xiong, 2011), this result also suggest that HBx can form CRL4A and CRL4B complexes in both subcellular compartments.

To identify potential substrates of HBx, we compared the list of proteins and looked for those that were identified exclusively, or with increased abundance, in the MLN4924-treated samples relative to the DMSO controls. While similar amounts of DDB1, CUL4A, and CUL4B proteins were present irrespective of MLN4924 treatment, a relatively small number of proteins that exhibited this behavior were identified (Figure 1B). Notably, among these candidates were four separate subunits of a single protein complex, structural maintenance of chromosomes 5/6 (SMC5/6) - SMC5, SMC6, NSMCE4A and NDNL2 - as well as other proteins implicated in antiviral defense, including NLRC4, DDX5, and DHX9.

To determine which, if any, of these candidates were substrates of CRL4<sup>HBx</sup> ligase, we compared the stability of each candidate in the absence and presence of HBx. HepG2 cells inducibly expressing HBx (Figure S1D–E) were treated with doxycycline for five days to activate HBx expression, and cells were then analyzed by western blot with antibodies recognizing the endogenous proteins. Most proteins were unchanged in the presence of HBx, but three of the tested proteins, SMC6, SMC5 and NSMCE4A, showed a clear decrease in abundance, suggesting that SMC5/6 may be a substrate of HBx (Figure 1C). To confirm the interaction of HBx with SMC5/6 proteins, we examined whether HBx binds to SMC5/6 by IP-western analysis after inducing the expression of 3xFLAG-HBx for 24 hours in transiently transfected HEK293T cells. In addition to DDB1, both ectopically expressed SMC5 and SMC6 were detected in the HBx immunoprecipitate (Figure 1D). Using antibodies that recognize SMC5/6, we observed that endogenous SMC5/6 was also bound and degraded by HBx in HepG2 cells after induction of HBx expression (Figure 1E). This degradation was dose-dependent (Figure 2A), and occurred within 2 days of HBx induction (Figure 2B).

### **HBV infection degrades SMC5/6 proteins in human hepatocyte cell lines and in HBV-infected human liver tissue in humanized mice in vivo**

We next used HepG2 cells expressing the HBV receptor sodium taurocholate cotransporting polypeptide (NTCP) to determine the activity of HBx in promoting SMC5/6 degradation in cells infected with HBV (Yan et al., 2012). We detected reduced levels of SMC6 in HBV+ cells by co-staining HBV core and SMC6 (Figure 2E). Importantly, we also observed greatly reduced levels of SMC5/6 in the human liver tissue of HBV-infected NRG Fah humanized mice (Figure 2F; (Li et al., 2014)). Together, these results indicate that HBx induces SMC5/6 degradation in HBV-infected cells.

### **HBx degrades SMC5/6 in a CRL4<sup>HBx</sup>- and proteasome-dependent manner**

To determine whether HBx regulates SMC5/6 degradation via the CRL4<sup>HBx</sup> E3 ligase, we examined the effect of knocking down DDB1, CUL4A or CUL4B on HBx-induced SMC5/6 degradation (Figure 2C). Knockdown of either DDB1 or a combination of CUL4A and CUL4B completely blocked HBx-induced SMC5/6 degradation, and individual knockdown of either CUL4A or CUL4B, which are functionally redundant, partially blocked SMC5/6 degradation. Further, this degradation was sensitive to the proteasome inhibitor MG132 (Figure 2D). These results suggest that CRL4 is the principle E3 ligase for HBx-promoted SMC5/6 degradation, which occurs by a proteasome-dependent pathway. Interestingly, while cells form three structurally and functionally similar heterodimeric SMC complexes (Jeppsson et al., 2014; Menolfi et al., 2015), only SMC5/6, but not cohesin (SMC1/3) or condensin (SMC2/4), was degraded by HBx, suggesting a unique function of SMC5/6 in antagonizing HBV.

### **HBx targets SMC5/6 for ubiquitylation by CRL4<sup>HBx</sup> E3 ligase**

To determine whether HBx promotes SMC5/6 degradation by catalyzing their polyubiquitylation, we first examined the effect of HBx on SMC5/6 in an *in vivo* ubiquitylation assay. HA-Ubiquitin, 3xFLAG-HBx and either myc-SMC5 or myc-SMC6 were co-transfected into HEK293T cells, and SMC5/6 proteins were isolated by myc

antibody pull-down. Both SMC5 and SMC6 showed a strong polyubiquitin ladder that was dependent upon expression of HBx, and this effect was reduced when HBx(R96E), a CRL4-binding deficient mutant, was used instead. (Figure 3A, S2A). SMC5/6 polyubiquitylation *in vivo* was clearly reduced by the knockdown of either CUL4A or CUL4B and nearly abolished by the knockdown of DDB1 (Figure 3B). Endogenously expressed SMC6 protein exhibited little ubiquitylation, but it was actively ubiquitylated by a K48-linked polyubiquitin chain upon expression of HBx (Figure 3C). Together, these results demonstrate that SMC5/6 is aberrantly ubiquitylated *in vivo* by the CRL4<sup>HBx</sup> E3 ligase, which catalyzes K48-linked polyubiquitylation of SMC5/6, thereby promoting their degradation by the proteasome pathway.

To investigate whether CRL4<sup>HBx</sup> E3 complex catalyzes direct polyubiquitylation of SMC5/6, we performed *in vitro* ubiquitylation assays using purified components. A robust ubiquitylation of SMC5 (Figure 3D and Figure S2B) and SMC6 (Figure 3E and Figure S2B) was observed when either protein was incubated with a mixture of immunopurified CUL4A and CUL4B E3 complex, immunopurified HBx protein, recombinant E1, E2 and ubiquitin, and ATP. A drop-out assay demonstrated that SMC5/6 polyubiquitylation is dependent on E1, E2, E3 and HBx. Again, substitution of wildtype HBx with DDB1-binding deficient R96E mutant HBx substantially reduced the polyubiquitylation of both SMC5 and SMC6, providing additional evidence that HBx bridges SMC5/6 to the DDB1-CUL4A/B-ROC1 E3 ligases for ubiquitylation.

### HBx counteracts SMC5/6 activity to enhance HBV gene expression

The principal role of HBx in HBV infection and pathogenesis is to activate transcription from the HBV cccDNA template. Since HBx requires DDB1-CRL4 binding for this function, and SMC5/6 is an HBx-CRL4 substrate, we next tested whether SMC5/6 degradation played an inhibitory role in HBV cccDNA activity. For this purpose, we have developed a minicircle HBV (mcHBV) cccDNA reporter system to measure this activity (Figure S1F–G and (Guo et al., 2016)). In this system, minicircle HBV cccDNA with no residual bacterial plasmid DNA is generated by a site-specific recombination reaction in bacteria (Kay et al., 2010). When transfected into HepG2 cells, this mcHBV cccDNA is packaged into a minichromosome, produces all HBV proteins, and can generate HBV virions (Li et al., submitted; and (Guo et al., 2016)). To facilitate monitoring of HBV gene expression, we have further cloned Gaussia luciferase into the mcHBV DNA under the control of the HBV core promoter. As is true for cccDNA during infection, transcription from the reporter mcHBV-GLuc DNA is strongly dependent upon HBx (Figure S1I). Transfection of mcHBV-GLuc reporter cccDNA produced highly stable luciferase activity (Figure S1H), whereas transfection of an HBx-deficient form of the mcHBV-GLuc cccDNA showed over a 4-fold decrease in activity, which could be rescued back to wild type levels by expression of HBx *in trans* (Figure S1I).

If HBx-mediated destruction of SMC5/6 promotes transactivation of mcHBV-Gluc cccDNA, then experimental depletion of SM5/6 should rescue HBx-deficiency. To test this, mcHBV reporter cccDNA with a mutant HBx gene ( X) was transfected into inducible HepG2-HBx cells. In the absence of HBx induction, gene expression activity from the X mcHBV

cccDNA was very low, but could be rescued by HBx induction (Figure 4A). When SMC6 or SMC5 was knocked down by shRNA transduction, gene expression from the HBx mutant mcHBV-Gluc was completely rescued, indicating that targeting SMC5/6 is likely a primary cause of HBx-mediated transactivation of mcHBV cccDNA. This effect was observed with two separate shRNAs targeting SMC6 or SMC5 (Figure 4A, Fig S3A–B), and it could be partially prevented by exogenous expression of shRNA-resistant SMC6 bearing silent mutations in the shRNA target sequence (Figure S3C, S3D), suggesting that this effect is specific to SMC5/6 function. In addition, we created a dominant negative form of SMC6 with a K to E point mutation in the ATPase active site (K82E) that prevents DNA binding and SMC5/6 function (Kanno et al., 2015). Expression of the SMC6(K82E) dominant negative mutant also resulted in an increase in mcHBV-Gluc gene expression (Figure 4B). These results show that SMC5/6 inhibits HBV gene expression from the HBV cccDNA template and that this function of SMC5/6 is antagonized by HBx.

Given that SMC6 knockdown could effectively rescue HBx-deficient mcHBV cccDNA, we next asked to what extent HBx could further stimulate mcHBV-Gluc gene expression in cells depleted of SMC6. Doxycycline was added to the cells to induce HBx expression, and luciferase activity was measured. Compared with un-induced cells, HBx-induced transactivation was 5.5-fold in control cells, but only about 1.7-fold in SMC6 knockdown cells (Figure S3E–G), indicating that SMC6 knockdown and HBx expression were performing overlapping functions. The residual transactivation of HBx in the presence of shRNA targeting SMC6 may be the result of incomplete SMC6 depletion, though we cannot exclude the possibility that HBx may have additional, SMC5/6-independent activities in activating transcription from the mcHBV cccDNA template. Nevertheless, these results indicate that HBx-mediated transactivation of mcHBV-Gluc gene expression is due in a large part to its activity in degrading SMC5/6.

### **SMC5/6 knockdown rescues infectivity of HBV X mutant virus in human hepatocytes**

HBx is essential for productive HBV infection and HBV X mutant has impaired replicative activity in target cells. To further test whether HBx-targeting of SMC5/6 represents the primary function of HBx in enhancing HBV infection and replication, we next asked whether SMC5/6 knockdown in NTCP+ HepG2 cells could rescue infection of HBx defective viruses. HepG2-NTCP cells can support HBV infection, as incubation with HBV results in subsequent production of HBsAg and HBeAg that was sensitive to the HBV entry inhibitor cyclosporin A (Figure S4A–C). We prepared a set of HepG2-NTCP cell lines transduced with shSMC6, shSMC5, or a non-targeting control shRNA (Figure S4G). We then infected these cells with either wild type HBV or HBV X mutant. HBeAg, HBsAg and HBV DNA were then monitored to measure HBV infection and replication (Figure 4C–F, S4). Wild type HBV was able to infect all of the cell lines with similar efficiency, with only a modest increase in the SMC5 and 6 knockdown cells (Figure 4C). HBV X mutant, on the other hand, failed to produce detectable levels of HBV replication in mock-transduced (parental) or shCtrl cells. This defect was efficiently rescued by SMC6 or SMC5 knockdown, which resulted in levels of HBV replication (HBeAg levels) that nearly matched those of wild type virus (Figure 4D). These findings were confirmed by measuring HBV HBsAg or HBV genomic DNA in the supernatant (Figure 4E and 4F, and Figure S4D–F).

Therefore, SMC5/6 knockdown and HBx expression were almost completely redundant with respect to enhancing HBV infection, suggesting that the principal function of HBx is to degrade the SMC5/6 complex and counteract its antiviral function.

## DISCUSSION

HBx activates HBV viral gene expression from the HBV cccDNA through a poorly understood mechanism (Slagle and Bouchard, 2016). HBx binds the DDB1 and is proposed to target a host factor for degradation by the DDB1-CUL4-ROC1 (CRL4) E3 ligase (Leupin et al., 2005; Li et al., 2010; Martin-Lluesma et al., 2008). Here, we identified the structural maintenance of chromosomes (SMC) complex proteins SMC5/6 as the substrates of CRL4<sup>HBx</sup>. Importantly, SMC5/6 complex was degraded by HBx in human hepatocytes expressing HBx or infected by HBV. We demonstrate that SMC5/6 knockdown, or inhibition with a dominant-negative SMC6, enhanced HBx-deficient mcHBV-Gluc gene expression, indicating that SMC5/6 is a restriction factor of the HBV cccDNA and is counteracted by HBx. When tested in NTCP+ human hepatocyte cells that support HBV infection, SMC5/6 knockdown rescued replication of HBx-deficient HBV virus, but had little effect on wild type HBV virus. These results indicate that a primary function of HBx is to ubiquitylate and degrade SMC5/6, which inhibits HBV replication by inhibiting HBV cccDNA activity. The results thus reveal a mechanism for HBx function in HBV-infected cells and provide a potential avenue for targeting HBV cccDNA function by blocking HBX-targeted, CRL4-catalyzed SMC5/6 ubiquitylation.

We demonstrate the functional significance of HBx-targeted SMC5/6 degradation on HBV replication using a physiologically relevant minicircle HBV cccDNA system, which is devoid of all bacterial plasmid DNA and in HBV cccDNA form (Kay et al., 2010). The minicircle HBV cccDNA-based assay depended on HBx for maximal activity and allowed us to study the effect of SMC5/6 and HBx on cccDNA activity in human hepatocyte cell lines in the absence of other forms of HBV genomes (Guo et al., 2016). We demonstrate that SMC5/6 knockdown, or inhibition with a dominant-negative SMC6, enhanced HBx-defective mcHBV-Gluc gene expression. When tested in NTCP+ human hepatocyte cells that support HBV infection, SMC5/6 knockdown rescued replication of HBx-deficient HBV virus, but had little effect on wild type HBV virus. Thus SMC5/6 is a major restriction factor of the HBV cccDNA that is counteracted by HBx.

Eukaryotic cells include three functionally related, but non-redundant SMC complexes - cohesins, condensins and the SMC5/6 complex - each consisting of SMC heterodimers and additional non-SMC subunits (Jeppsson et al., 2014). SMC complexes play a central role in controlling multiple chromatin processes, such as sister chromatid cohesion, chromosome condensation, DNA replication, DNA repair and transcription (Jeppsson et al., 2014). These functions bear a striking overlap with some of the multiple reported HBx activities. For example, HBx, via its DDB1-binding activity, impairs DNA repair (Becker et al., 1998), S phase progression, chromosome segregation and mitosis (Martin-Lluesma et al., 2008). Our finding that HBx degrades SMC5/6 offers a plausible explanation to these cellular defects previously linked to HBx. We also note that HBx selectively interacts with and targets the degradation of SMC5/6, but not the other two SMC complexes, suggesting a unique function

of SMC5/6 in inhibiting HBV. This is consistent with the observations that each SMC complex also has its distinct functions. While cohesins and condensins are required for establishing links between sister chromatids and for chromatin compaction, SMC5/6 plays a role in chromosome segregation and repair. Loss of SMC5/6 function impairs genome stability and resolution of replication-induced DNA supercoiling (Kegel et al., 2011). HBx has been shown to be a cofactor in HCC development. We speculate that dysregulation of DNA repair and induction of chromosomal instability by the expression of HBx may contribute to the HBV-mediated HCC development.

It will be important to determine how SMC5/6 inhibits cccDNA function. With its known functions in modulating chromatin structure and function, it is likely that SMC5/6 suppresses gene expression from episomal DNA such as HBV cccDNA via epigenetic mechanisms, and HBx has evolved to counteract SMC5/6 activity to facilitate HBV replication. The major limitation of current HBV therapies is the inability to target cccDNA. SMC5/6 possesses intrinsic DNA binding activity and was recently shown to function as an intermolecular linker, bridging DNA molecules through topological entrapment (Kanno et al., 2015). This could inhibit cccDNA function by promoting direct compaction of the cccDNA minichromosome or by tethering it to inactive or heterochromatic regions of the nucleus. It will be of great interest to determine exactly how SMC5/6 inhibits HBV cccDNA function, which could uncover new therapeutic opportunities for targeting HBV cccDNA and other viruses with episomal DNA genomes.

A paper reporting the same finding has very recently been published (Decorsiere et al., 2016). Although the two studies are consistent in the main finding about HBx targeting SMC5/6, our study additionally contains two important aspects. First, we provide evidence that SMC5/6 is a direct ubiquitylation target of HBx-DDB1-CRL4 E3 ligase. Second, we show that of three functionally related SMC heterodimeric complexes, only SMC5/6, but not cohesin or condensin complexes, are targeted by HBx.

## EXPERIMENTAL PROCEDURES

### Immunoprecipitation-Mass spectrometry

HepG2-HBx-FSH8 cells were induced with 120 ng/ml doxycycline for 24 hours, treated with MLN4924 (1 $\mu$ M, Cayman Chemical) or DMSO for 4 hours, and lysed in NP-40 Lysis Buffer (0.5% Nonidet P-40, 50 mM Tris pH 7.5, 150 mM NaCl) with Halt protease inhibitor (Pierce) and DMSO or MLN4924 on ice after culturing. Flag-SBP-HBx was precipitated with anti-Flag M2 resin (Sigma) affinity eluted with 200 $\mu$ g/ml 3x FLAG peptide (Sigma). Eluates were then incubated with streptavidin agarose (GE Life Sciences). Beads were washed 4 times with lysis buffer, and peptides were generated by on-beads trypsin digestion and isolated by filter-aided sample preparation (FASP). LCMS was performed as described (Yu and Pieper, 2015). Database search was conducted using SEQUEST and Proteome Discoverer (Yu et al., 2014). Spectral counts were compared between samples, and those proteins with 4 fold increase over negative control runs and increased after MLN4924 treatment were selected as potential HBx substrates. Anti-FLAG immunoprecipitations for IP western analysis were performed as above, but eluted at 70°C for 20 minutes in SDS-PAGE loading buffer.



## HBV infection

HepAD38 cells (kindly provided by Dr. J. Hu and Dr. C. Seeger) grown without doxycyclin, or HepG2 cells transiently transfected with a plasmid encoding HBV X (Dr. J. Hu), were grown for 5–7 days (Ladner et al., 1997). Media was clarified by centrifugation at  $10,000 \times g$ . Supernatants were supplemented with 10% PEG-8000, incubated at 21°C for 30 min and 4°C for 60 min, and centrifuged again. The pellet containing viral particles was resuspended in 1/100 the original volume with serum-free media and flash frozen.

For HBV infection, HepG2-NTCP cells (Yan et al., 2012) were plated at 70–80% confluency. After 24 hours, media was supplemented with 4% PEG-8000. HBV was added at  $4 \times 10^3$  or  $4 \times 10^4$  genome equivalents per cell. After 16 hrs, cells were rinsed  $4 \times$  in PBS and cultured in media with 2% DMSO. Media was collected and changed every 48 hours.

## Detection of HBV replication by ELISA

HBsAg and HBeAg were detected using kits from Alpha diagnostics and Abnova, respectively.

## Detection of HBc and SMC6 by immunofluorescence co-staining

HBV infected cells were seeded onto cover glasses 9–12 days after infection. After 24 hours, cells were fixed in 4% paraformaldehyde in PBS, rinsed in PBS, permeabilized in 0.15% TritonX-100, and then washed 3 times in PBS. Blocking buffer (0.2% BSA, 5% Donkey Serum, 0.1% Triton-X100, 1xPBS, pH 7.4) was added for 30 minutes to block nonspecific binding. Primary antibodies targeting HBc (Zeta) or SMC6 (Abgent) were diluted in blocking buffer at 1:200 and 1:400, respectively, and incubated with cells for 60 minutes. Cells were washed  $3 \times$  in blocking buffer and then incubated for 60 minutes with donkey anti-rabbit Alexa 488 and donkey anti-mouse Alexa 594 secondary antibodies (1:1000, Life Technologies). The cover glasses were washed in blocking buffer, in PBS, rinsed quickly in ddH<sub>2</sub>O, and mounted with fluorescence mounting media with DAPI (Abcam).

## Luciferase assays

Cells were transfected with mcHBV-Gluc DNA and carrier DNA (pUC19). The next day, cells were transduced with lentiviruses expressing shRNA or proteins. Alternatively, cells were cotransfected with mcHBV-Gluc DNA and the relevant constructs. Media was then collected every 2 days and assessed for luciferase activity on a GLOMAX microplate luminometer (Promega).

## Statistical Analysis

Comparisons were performed using t-test or ANOVA with Tukey's post-hoc test using Graphpad Prism software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## REFERENCES

- Angers S, Li T, Yi X, MacCoss MJ, Moon RT, Zheng N. Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. *Nature*. 2006; 443:590–593. [PubMed: 16964240]
- Becker SA, Lee TH, Butel JS, Slagle BL. Hepatitis B virus X protein interferes with cellular DNA repair. *J Virol*. 1998; 72:266–272. [PubMed: 9420223]
- Cha CK, Kwon HC, Cheong JY, Cho SW, Hong SP, Kim SO, Yoo WD. Association of lamivudine-resistant mutational patterns with the antiviral effect of adefovir in patients with chronic hepatitis B. *J Med Virol*. 2009; 81:417–424. [PubMed: 19152409]
- Seeger, Christoph; Zoulim, Fabien; Mason, WS. *Fields Virology* (5 th). 2007
- Decorsiere A, Mueller H, van Breugel PC, Abdul F, Gerossier L, Beran RK, Livingston CM, Niu C, Fletcher SP, Hantz O, et al. Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature*. 2016; 531:386–389. [PubMed: 26983541]
- Dienstag JL. Hepatitis B virus infection. *N Engl J Med*. 2008; 359:1486–1500. [PubMed: 18832247]
- Emanuele MJ, Elia AE, Xu Q, Thoma CR, Izhar L, Leng Y, Guo A, Chen YN, Rush J, Hsu PW, et al. Global identification of modular cullin-RING ligase substrates. *Cell*. 2011; 147:459–474. [PubMed: 21963094]
- Guo X, Chen P, Hou X, Xu W, Wang D, Wang TY, Zhang L, Zheng G, Gao ZL, He CY, et al. The recombinant cccDNA produced using minicircle technology mimicked HBV genome in structure and function closely. *Scientific reports*. 2016; 6:25552. [PubMed: 27174254]
- He YJ, McCall CM, Hu J, Zeng Y, Xiong Y. DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases. *Genes Dev*. 2006; 20:2949–2954. [PubMed: 17079684]
- Higa LA, Wu M, Ye T, Kobayashi R, Sun H, Zhang H. CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol*. 2006; 8:1277–1283. [PubMed: 17041588]
- Hodgson AJ, Hyser JM, Keasler VV, Cang Y, Slagle BL. Hepatitis B virus regulatory HBx protein binding to DDB1 is required but is not sufficient for maximal HBV replication. *Virology*. 2012; 426:73–82. [PubMed: 22342275]
- Jackson S, Xiong Y. CRL4s: the CUL4-RING E3 ubiquitin ligases. *Trends Biochem Sci*. 2009; 34:562–570. [PubMed: 19818632]
- Jeppsson K, Kanno T, Shirahige K, Sjogren C. The maintenance of chromosome structure: positioning and functioning of SMC complexes. *Nature reviews Molecular cell biology*. 2014; 15:601–614. [PubMed: 25145851]
- Jin J, Arias EE, Chen J, Harper JW, Walter JC. A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Molecular cell*. 2006; 23:709–721. [PubMed: 16949367]
- Kanno T, Berta DG, Sjogren C. The Smc5/6 Complex Is an ATP-Dependent Intermolecular DNA Linker. *Cell reports*. 2015; 12:1471–1482. [PubMed: 26299966]
- Kay MA, He CY, Chen ZY. A robust system for production of minicircle DNA vectors. *Nat Biotechnol*. 2010; 28:1287–1289. [PubMed: 21102455]
- Keasler VV, Hodgson AJ, Madden CR, Slagle BL. Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo. *J Virol*. 2007; 81:2656–2662. [PubMed: 17182675]
- Kegel A, Betts-Lindroos H, Kanno T, Jeppsson K, Strom L, Katou Y, Itoh T, Shirahige K, Sjogren C. Chromosome length influences replication-induced topological stress. *Nature*. 2011; 471:392–396. [PubMed: 21368764]

- Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, Seeger C, King RW. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. *Antimicrob Agents Chemother.* 1997; 41:1715–1720. [PubMed: 9257747]
- Lee J, Zhou P. DCAFs, the missing link of the CUL4-DDB1 ubiquitin ligase. *Molecular cell.* 2007; 26:775–780. [PubMed: 17588513]
- Lee TH, Elledge SJ, Butel JS. Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. *J Virol.* 1995; 69:1107–1114. [PubMed: 7815490]
- Leupin O, Bontron S, Schaeffer C, Strubin M. Hepatitis B virus X protein stimulates viral genome replication via a DDB1-dependent pathway distinct from that leading to cell death. *J Virol.* 2005; 79:4238–4245. [PubMed: 15767425]
- Li F, Cowley DO, Banner D, Holle E, Zhang L, Su L. Efficient genetic manipulation of the NOD-Rag1<sup>-/-</sup> IL2RgammaC-null mouse by combining in vitro fertilization and CRISPR/Cas9 technology. *Scientific reports.* 2014; 4:5290. [PubMed: 24936832]
- Li F, Nio K, Murphy CMM, Su L. Studying HBV infection and therapy in immune deficient NOD-Rag1<sup>-/-</sup> IL2RgammaC-null (NRG) fumarylacetoacetate hydrolase (Fah) knockout mice transplanted with human hepatocytes. *Methods in molecular biology.* 2015
- Li T, Robert EI, van Breugel PC, Strubin M, Zheng N. A promiscuous alpha-helical motif anchors viral hijackers and substrate receptors to the CUL4-DDB1 ubiquitin ligase machinery. *Nat Struct Mol Biol.* 2010; 17:105–111. [PubMed: 19966799]
- Martin-Lluesma S, Schaeffer C, Robert EI, van Breugel PC, Leupin O, Hantz O, Strubin M. Hepatitis B virus X protein affects S phase progression leading to chromosome segregation defects by binding to damaged DNA binding protein 1. *Hepatology.* 2008; 48:1467–1476. [PubMed: 18781669]
- Menolfi D, Delamarre A, Lengronne A, Pasero P, Branzei D. Essential Roles of the Smc5/6 Complex in Replication through Natural Pausing Sites and Endogenous DNA Damage Tolerance. *Molecular cell.* 2015; 60:835–846. [PubMed: 26698660]
- Nakagawa T, Xiong Y. X-linked mental retardation gene CUL4B targets ubiquitylation of H3K4 methyltransferase component WDR5 and regulates neuronal gene expression. *Molecular cell.* 2011; 43:381–391. [PubMed: 21816345]
- Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. *Gut.* 2015
- Revill P, Testoni B, Locarnini S, Zoulim F. Global strategies are required to cure and eliminate HBV infection. *Nature reviews Gastroenterology & hepatology.* 2016; 13:239–248.
- Scaglione SJ, Lok AS. Effectiveness of hepatitis B treatment in clinical practice. *Gastroenterology.* 2012; 142:1360–1368. e1361. [PubMed: 22537444]
- Sitterlin D, Lee TH, Prigent S, Tiollais P, Butel JS, Transy C. Interaction of the UV-damaged DNA-binding protein with hepatitis B virus X protein is conserved among mammalian hepadnaviruses and restricted to transactivation-proficient X-insertion mutants. *J Virol.* 1997; 71:6194–6199. [PubMed: 9223516]
- Slagle BL, Bouchard MJ. Hepatitis B Virus X and Regulation of Viral Gene Expression. *Cold Spring Harb Perspect Med.* 2016; 6
- Tan MK, Lim HJ, Bennett EJ, Shi Y, Harper JW. Parallel SCF adaptor capture proteomics reveals a role for SCFFBXL17 in NRF2 activation via BACH1 repressor turnover. *Molecular cell.* 2013; 52:9–24. [PubMed: 24035498]
- Tang H, Delgermaa L, Huang F, Oishi N, Liu L, He F, Zhao L, Murakami S. The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. *J Virol.* 2005; 79:5548–5556. [PubMed: 15827169]
- Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *elife.* 2012; 1:e00049. [PubMed: 23150796]
- Yu Y, Pieper R. Urinary pellet sample preparation for shotgun proteomic analysis of microbial infection and host-pathogen interactions. *Methods in molecular biology.* 2015; 1295:65–74. [PubMed: 25820714]

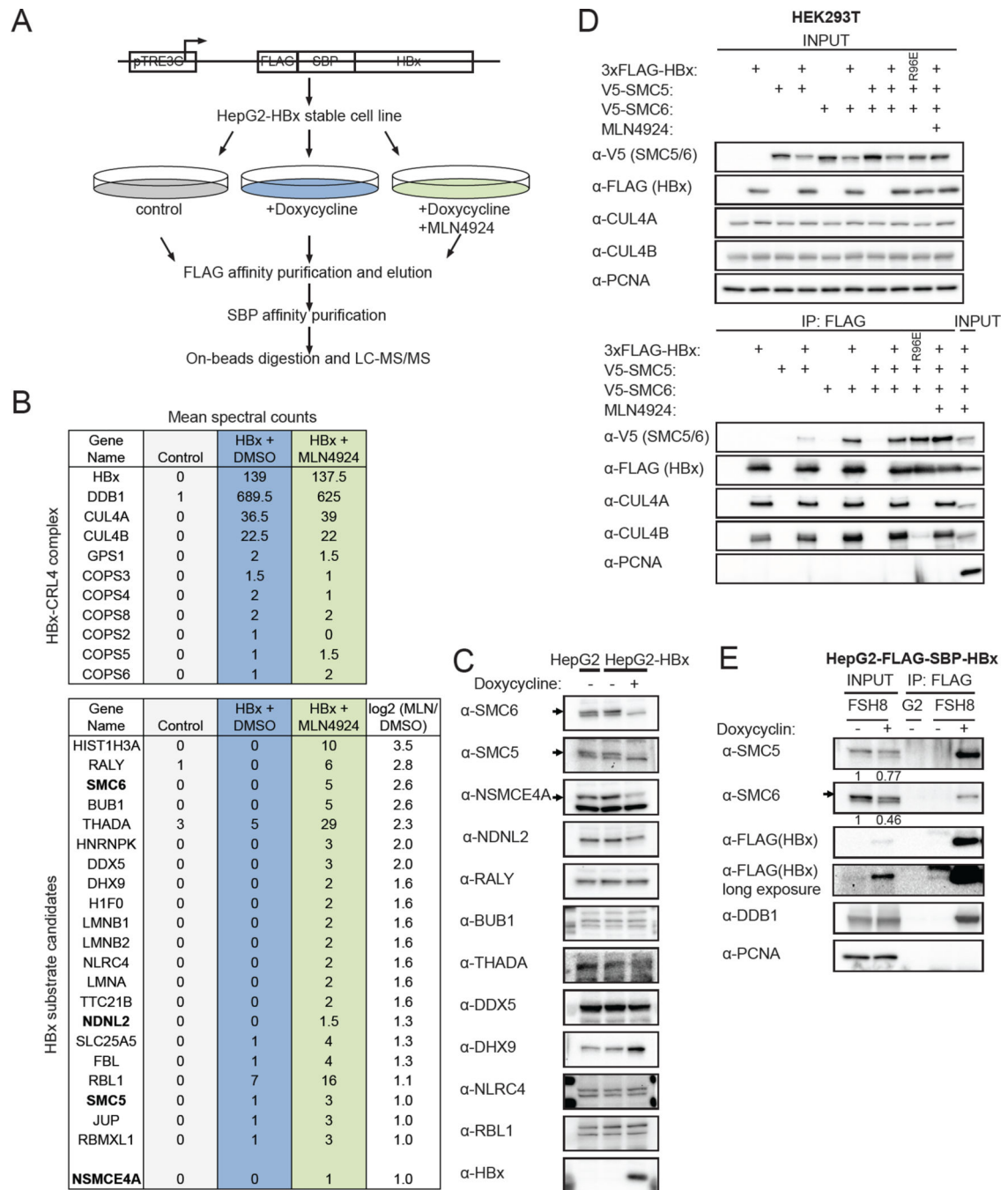
- Yu Y, Suh MJ, Sikorski P, Kwon K, Nelson KE, Pieper R. Urine sample preparation in 96-well filter plates for quantitative clinical proteomics. *Analytical chemistry*. 2014; 86:5470–5477. [PubMed: 24797144]
- Zeisel MB, Lucifora J, Mason WS, Sureau C, Beck J, Levrero M, Kann M, Knolle PA, Benkirane M, Durantel D, et al. Towards an HBV cure: state-of-the-art and unresolved questions-report of the ANRS workshop on HBV cure. *Gut*. 2015; 64:1314–1326. [PubMed: 25670809]

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**Figure 1. Identification of CRL4-HBx substrates by TAP/MS and CRL inhibition**

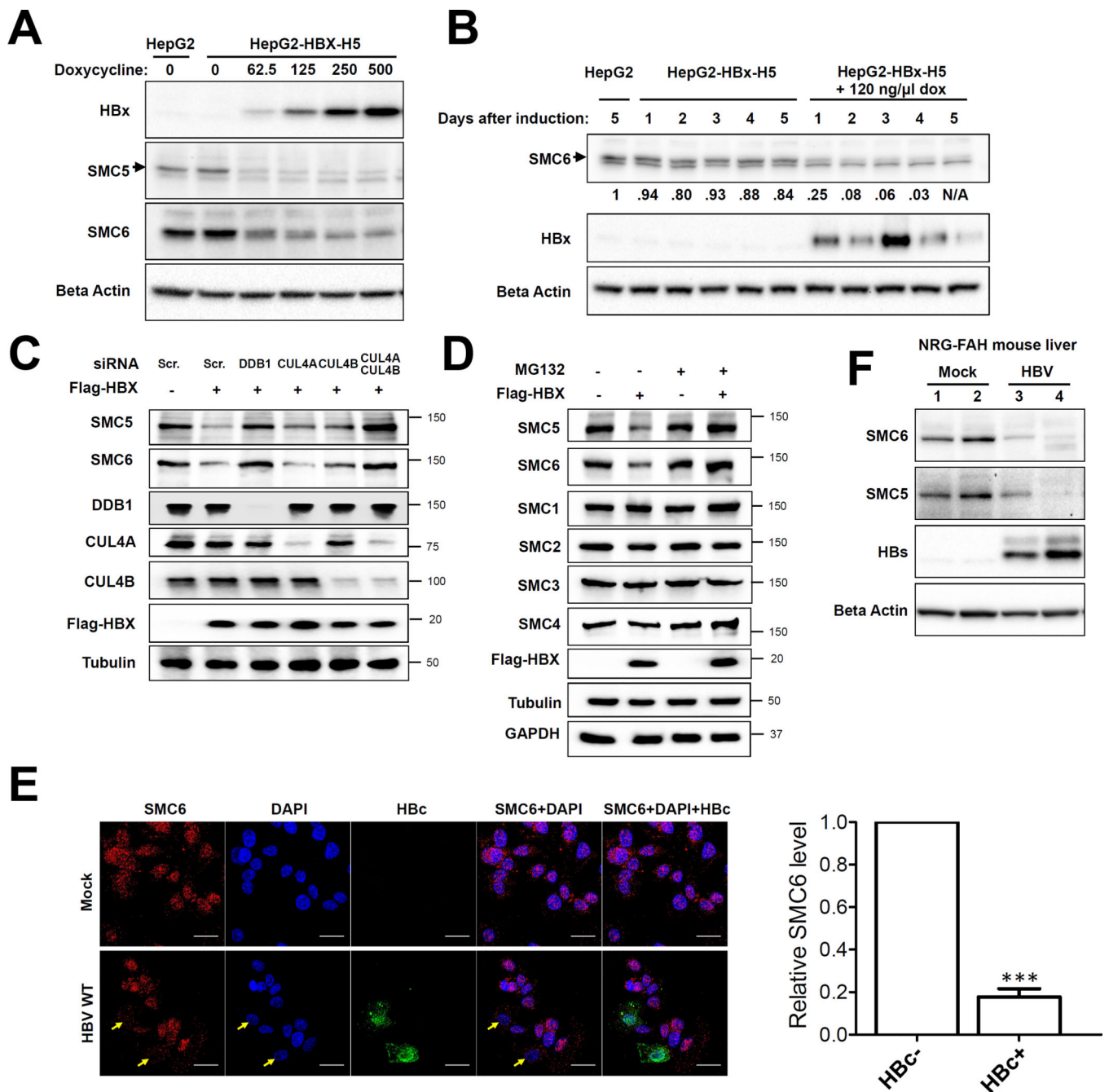
(A) Experimental design to identify the substrate of CRL4<sup>HBx</sup>. HepG2 cells inducibly expressing FLAG-SBP-HBx were induced with doxycycline and treated with MLN4924 to stabilize HBx-substrate interactions, which were then purified and analyzed by LC-MS/MS. (B) Spectral counts of proteins identified under each condition were compared to identify potential CRL4<sup>HBx</sup> substrates. Upper table: HBx interactions with the CRL4 E3 ligase. Lower table: Proteins identified either exclusively or in greater amounts with MLN4924 treatment relative to DMSO were shown as potential substrates. The table shows the

combined results from two separate experiments. For proteins identified in both experiments, the mean number of spectral counts is listed.

**(C)** Substrate candidates were screened for HBx-induced instability 5 days after induction of HBx expression with 500ng/ml doxycycline.

**(D)** HEK293T cells were cotransfected with constructs expressing 3xFLAG-HBx, V5-tagged SMC5/6, or empty vector controls. After 41 hours, cells were treated or not with 1  $\mu$ M MLN4924 and harvested 10 hours later. Total cell lysates (top) or FLAG immunoprecipitates (bottom) were then analyzed for the indicated proteins.

**(E)** HepG2 cells inducibly expressing FLAG-SBP-HBx (FSH8) were induced with 120 ng/ml doxycycline for 48 hours, and endogenous SMC5/6 was co-immunoprecipitated with HBx using anti-FLAG resin. Unmodified HepG2 cells (G2) were used as a negative control.



**Figure 2. HBx expression and HBV infection degrades SMC5/6**

(A, B) Inducible HepG2-HBx-H5 cells were treated with a range of doxycycline concentrations (A) or for different lengths of time (B), and SMC5 and SMC6 levels were analyzed by immunoblot.

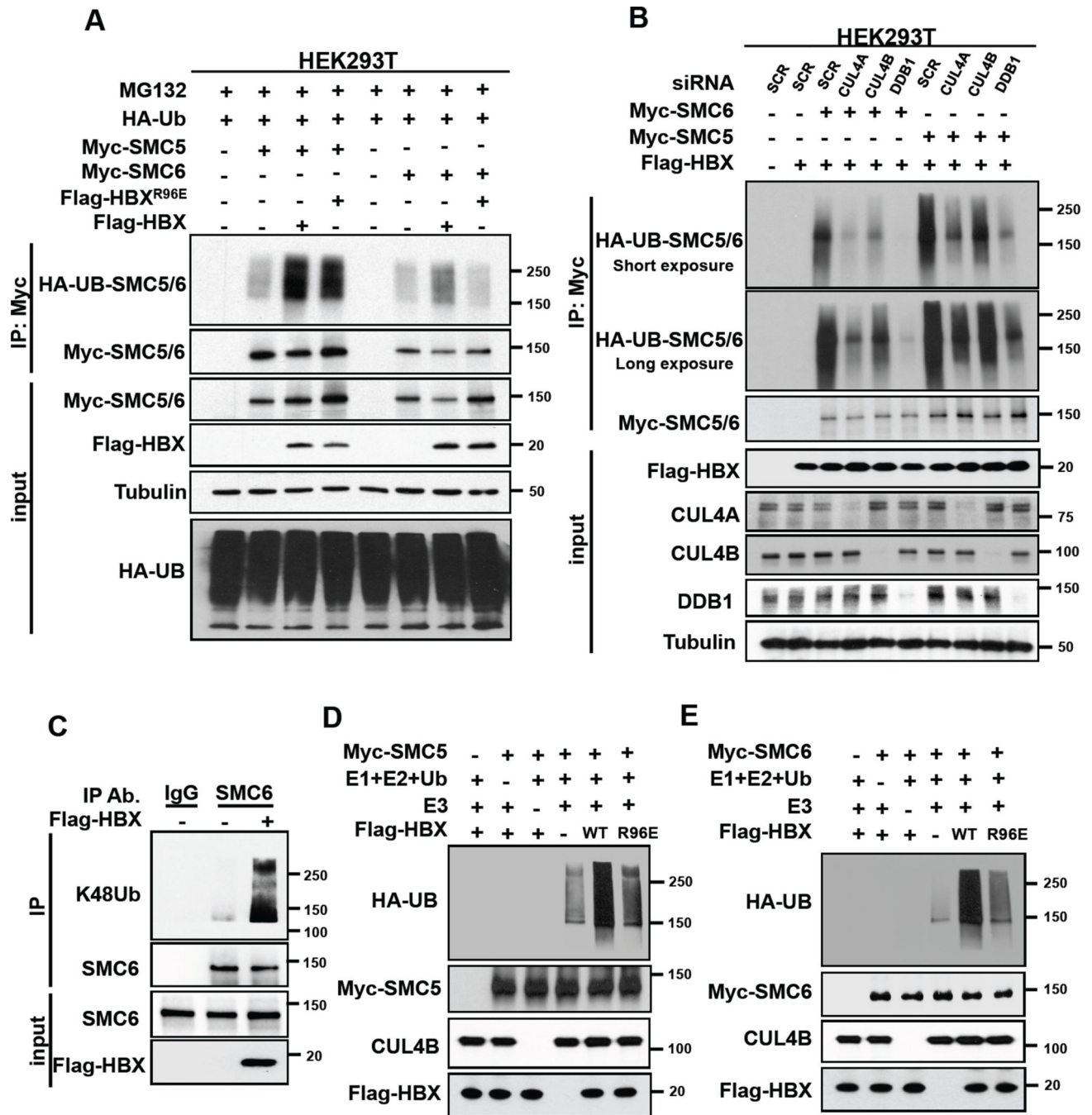
(C) HEK293T cells were first transfected with control siRNA or siRNA targeting indicated genes for 24 hours and transfected with Flag-HBx for another 24 hours, followed by immunoblot analysis.

(D) HEK293T cells were transfected with Flag-tagged HBx for 24 hours and then treated with MG132 (2 μM) for 24 hours, followed by cell lysis and SDS-PAGE.

(E) Confocal microscopy of mock- and HBV-infected human hepatocytes stained with SMC6 (red) and Hbc (green) antibodies and counterstained with DAPI. A representative view is shown, and arrows indicate cells where SMC6 is degraded by HBV infection. Scale bars = 20  $\mu\text{m}$ . Intensities of nuclear SMC6 fluorescence were quantified from Hbc+ and Hbc- cells of multiple fields using ImageJ software. The relative SMC6 level in each Hbc+ cells was normalized to that of Hbc- cells in the same view field. The average of relative SMC6 intensity was calculated from 18 Hbc+ and 18 Hbc- cells. \*\*\*\*,  $p < 0.0001$  (t-test).

(F) Liver samples from NRG-FAH-hu hepatocyte mice with human liver reconstitution (>60% human reconstitution) were analyzed for SMC6 levels by immunoblot in the presence or absence of HBV infection. HBV titers for mice #3 and #4 were  $7.47 \times 10^{11}$  and  $2.73 \times 10^8$  per ml, respectively.





**Figure 3. SMC5/6 is a direct ubiquitylation substrate of CRL4-HBx**

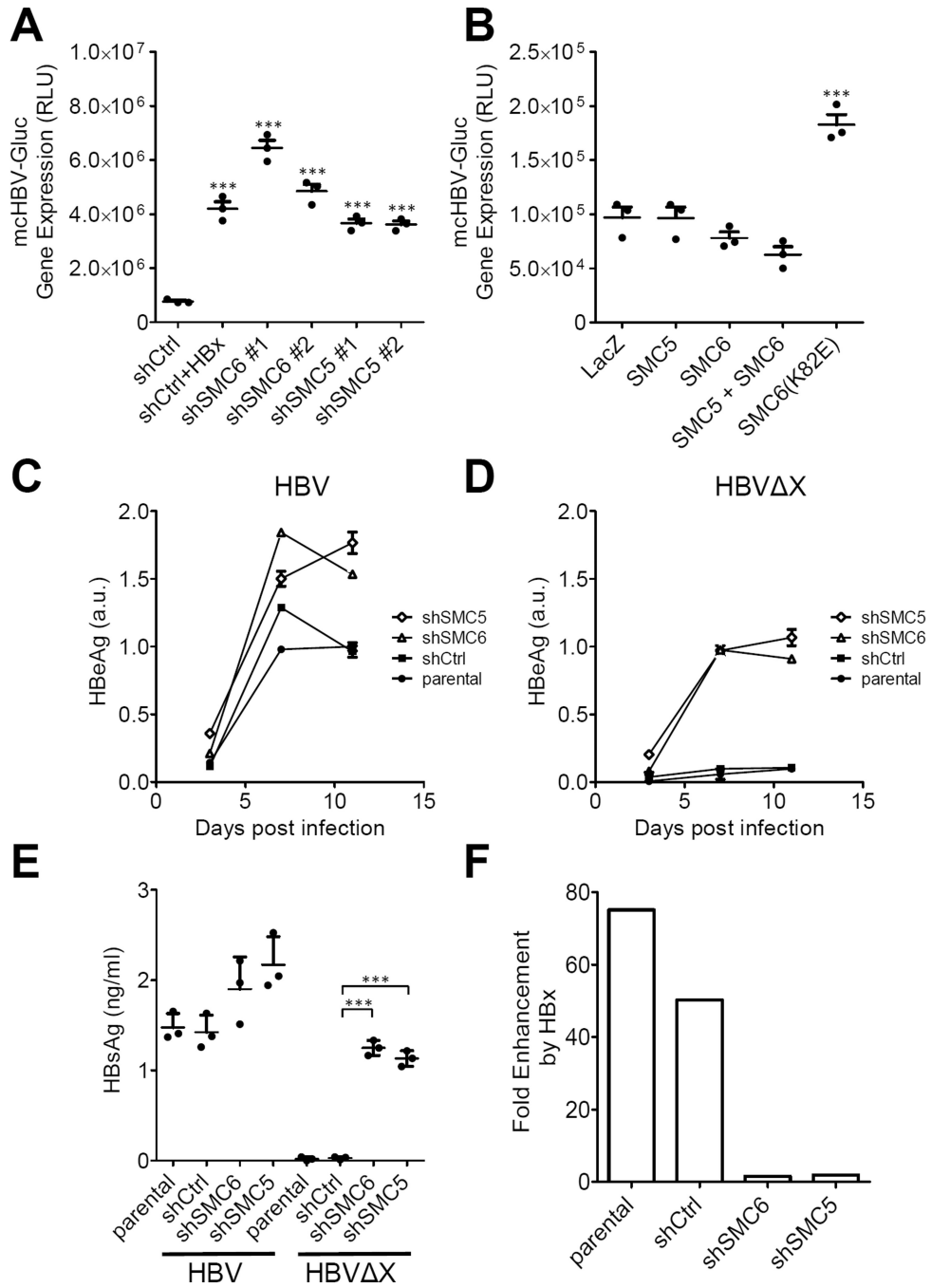
(A) Wild-type, but not DDB1-binding deficient R96E mutant, HBx promotes SMC5 and SMC6 polyubiquitylation in vivo. HEK293T cells were transfected with the indicated plasmids and treated with MG132 4h before harvest. Whole cell lysates were prepared under denaturing conditions and ubiquitylation of SMC5 and SMC6 were examined by coupled IP-western.

(B) Knockdown of DDB1, CUL4A or CUL4B inhibits HBx-promoted SMC5 and SMC6 polyubiquitylation in vivo. HEK293T cells were first transfected with indicated siRNA

oligonucleotides for 24h, then transfected with plasmids expressing the indicated proteins for another 48h and treated with MG132 4h before harvest. Knockdown was verified by immunoblotting with whole cell lysate. In vivo SMC5 or SMC6 ubiquitylation was determined by IP-western blot analysis under denaturing conditions.

**(C)** HEK293T cells were transfected with indicated plasmids and treated with MG132 before harvest. Whole cell lysates were prepared under denaturing conditions. Endogenous SMC6 was precipitated using a SMC6 antibody and the ubiquitylation was examined by western-blot using an antibody recognizing K48-linked polyubiquitin chain.

**(D, E)** Wild-type, but not DDB1-binding deficient R96E mutant, HBx promotes SMC5 and SMC6 polyubiquitylation by the CRL4 E3 ligase in vitro. Immunopurified SMC5 (C) or SMC6 (D) protein was incubated with a mixture of CUL4A and CUL4B immune-complexes and purified HBx in a buffer containing recombinant ubiquitin, E1, E2 and ATP. Reactions were terminated by addition SDS loading buffer, followed by SDS-PAGE and immunoblot with the indicated antibodies.



**Figure 4. HBx targets SMC5/6 to enhance HBV gene expression and HBV replication**  
**(A)** Knockdown of SMC5 or SMC6 enhances HBV X minicircle mcHBV-Gluc gene expression. HepG2-HBx-H5 cells were transfected with HBV X minicircle cccDNA, subcultured into 96 well plates, and then transduced with lentivirus encoding the shRNA indicated. Gaussia Luciferase activity in the culture supernatant was analyzed after 14 days. HBx expression was induced where indicated by the addition of doxycycline (400ng/ul).

**(B)** Expression of dominant-negative SMC6(K82E) rescues expression from HBV X mcHBV-Gluc cccDNA. HepG2-HBx-H5 cells were co-transfected with HBV X mcHBV-Gluc cccDNA and the indicated expression constructs. Luciferase was assayed after 8 days. **(C – F)** Knockdown of SMC5 or SMC6 in HepG2-NTCP cells enhanced replication of the HBV X mutant virus, but had little effect on wild type HBV virus. HepG2-NTCP cells were transduced with lentivirus expressing shRNA targeting SMC5, SMC6, or a non-target control sequence (shCtrl). After selection, transduced cells were infected with wild type or X HBV. Media was collected every other day, and HBeAg in the culture media was analyzed by ELISA at the times indicated (C–D). HBV replication was further confirmed by measuring HBsAg by ELISA at nine days post infection (E). \*\*\*,  $p < 0.001$ . The extent to which HBx promoted HBV replication in each cell line was calculated by dividing the HBsAg level from wt HBV infected cells by that from HBV X infected cells (F).