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Smc5/6 silences episomal transcription by a three-step function

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1. Extended Data

Figure #	Figure title One sentence only	Filename This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: <i>Smith_ED_Fig1.jpg</i>	Figure Legend If you are citing a reference for the first time in these legends, please include all new references in the main text Methods References section, and carry on the numbering from the main References section of the paper. If your paper does not have a Methods section, include all new references at the end of the main Reference list.
Extended Data Fig. 1	Smc5/6 is the only SMC complex to silence expression of the episomal HBV genome. Related to Figure 1.	NSMB-A45419_ED1.TIF	<p>a, PHH were transfected with a non-targeting control siRNA (siCtrl) or with siRNA targeting the indicated Smc subunits. Cells were then incubated for 3 days and infected with wild-type (WT) or an HBx-deficient (ΔX) HBV. HBeAg secretion, a marker for HBV gene expression, was measured 14 days later. HBeAg levels are expressed as a percentage of those in control cells infected with HBV(WT) (grey bars) or in siSmc6-treated cells infected with HBV(ΔX) (black bars). Data are means \pm SEM of three independent experiments with samples from one PHH donor. The schematic diagram summarizing the design of the study is shown at the top.</p> <p>b, The mRNA levels of the indicated Smc proteins in the samples analyzed in a were determined by real-time RT-PCR and normalized to β-actin. Values are expressed as a percentage of those in control cells (siCtrl). Data are means \pm SEM of three independent experiments with one PHH donor.</p> <p>c, Luciferase assay for the ChIP experiment presented in Fig. 1c. Data are means \pm SEM of 2 independent experiments.</p> <p>d, HA-Smc2 assembles into a condensin complex. Whole extracts prepared from HepG2 cells expressing the indicated HA-tagged SMC protein in a CRISPR/Cas9 knockout background were immunoprecipitated with anti-HA antibodies. The amounts recovered and the presence of Smc5, CAP-H and Nse4 in the eluates were assessed by Western blotting. CAP-H and Nse4 are the kleisin subunits of, respectively, condensin (Smc2) and the Smc5/6 complex. See Fig. 1a. Actin serves as a negative control. The experiment was repeated twice independently with similar results.</p>
Extended Data Fig. 2	Effect of single-subunit depletion on Smc5/6 complex integrity and restriction activity	NSMB-A45419_ED2.TIF	HepG2 cells were transduced with lentiviral constructs expressing Cas9 alone (Ctrl) or Cas9 together with sgRNA targeting the indicated Smc5/6 subunits, or with lentiviruses encoding GFP or GFP-HBx, as indicated. After selection, cells were transfected with a luciferase reporter plasmid. Luciferase assay and Western blot analysis were performed 5 days later. Hsp90 serves as a loading control. Unessential lanes were removed from the original blot images. Luciferase activity is relative to that measured

			in cells expressing HBx, which was set to 10. Data are means \pm SEM of 3 independent experiments.
Extended Data Fig. 3	Involvement of Smc5 and Smc6 ATP binding and hydrolysis in Smc5/6 episomal DNA binding and restriction, and degradation by HBx. Related to Figure 2.	NSMB-A45419_ED3.TIF	<p>a, Luciferase assay for the ChIP experiment presented in Fig. 2c. Data are means \pm SEM of 3 independent experiments.</p> <p>b, Smc6 ATP binding mutants normally assemble into Smc5/6 complexes. Whole extracts prepared from HepG2 cells depleted of Smc6 and expressing HA-GFP or the indicated HA-tagged SMC protein were immunoprecipitated with anti-HA antibodies. The amounts recovered and the presence of other Smc5/6 subunits in the eluates were assessed by Western blotting. Hsp90 serves as a negative control. The experiment was repeated twice independently with similar results.</p> <p>c, Luciferase assay for the ChIP experiment presented in Fig. 2d. Data are means \pm SEM of 3 independent experiments.</p> <p>d, HepG2 cells depleted of Smc6 and expressing GFP or the indicated wild-type or mutant Smc6 proteins from lentiviral vectors exactly as in Fig. 2b were also transduced with GFP or GFP-HBx. Western blot analysis was performed as before. GAPDH serves as a loading control. The experiment was repeated thrice independently with similar results.</p>
Extended Data Fig. 4	Nse4a performs an essential function in Smc5/6 restriction for which Nse4b cannot substitute. Related to Figure 3.	NSMB-A45419_ED4.TIF	<p>a, Schematic diagram of Nse4a (long), the shorter isoform of Nse4a, Nse4b and the Nse4b variant bearing the N-terminal region unique to Nse4a. White boxes indicate Nse4a sequences and the region of the short Nse4a protein common to both splicing isoforms. Hatched boxes indicate regions of Nse4b showing homology to Nse4a. The grey box indicates a region with no homology. Highlighted in black are the highly conserved N-terminal and C-terminal kleisin domains that have the potential to form helix-turn-helix and winged-helix motifs and are involved in Nse4 interaction with Smc6 and Smc5, respectively (Palecek et al., 2006; Vondrova et al., 2020).</p> <p>b, Luciferase assay for the ChIP experiment presented in Fig. 3b. Data are means \pm SEM of 2 independent experiments.</p> <p>c, Same experiment as in Fig. 3a but including an Nse4b chimeric protein carrying the N-terminal region unique to Nse4a (Nse4a-b; see panel a). Expression of Nse4b and Nse4a-b was inferred from their stabilization effect on the other Smc5/6 subunits (lane 8). Data are means \pm SEM of 2 independent experiments.</p>
Extended Data Fig. 5	Nse1 and Nse3 DNA-binding mutants are functional in vivo.	NSMB-A45419_ED5.TIF	a,b , Control HepG2 cells (black bars) and cells depleted of Nse1 (a) or Nse3 (b ; grey bars) were transfected with a luciferase reporter plasmid and shortly after transduced with GFP or the corresponding wild-type or DNA-binding

			mutant protein as indicated (Zabradý et al., 2016). Luciferase assay and Western blot analysis were as before. Data are means \pm SEM of 3 independent experiments.
Extended Data Fig. 6	HBx triggers Smc5/6 degradation in the absence of Nse2. Related to Figure 4.	NSMB-A45419_ED6.TIF	Extended Data Figure 6. HBx triggers Smc5/6 degradation in the absence of Nse2. Related to Figure 4. a , Control HepG2 cells (black bars) and cells depleted of Nse2 (grey bars) were transfected with a reporter gene and transduced with GFP or Flag-tagged Nse2 (F-Nse2). Cells were then split and further transduced with GFP or GFP-HBx. Luciferase assay and Western blot analysis were as before. Data are expressed as mean \pm SEM of 2 independent experiments. b , Luciferase assay for the ChIP experiment presented in Fig. 4b. Data are expressed as mean \pm SEM of 3 independent experiments. c , Single channel confocal images of middle right panels merged images presented in Fig. 4d.
Extended Data Fig. 7	HBx triggers Smc5/6 degradation in the absence of SLF2. Related to Figure 5.	NSMB-A45419_ED7.TIF	a , Control HepG2 cells (black bars) and cells depleted of SLF2 (grey bars) were transfected with a reporter gene and shortly after transduced with GFP, GFP-HBx and/or SLF2 in the indicated combinations. Luciferase assay and Western blot analysis were performed as before. Data are expressed as mean \pm SEM of 2 independent experiments. b , Luciferase assay for the ChIP experiment presented in Fig. 5b. Data are means \pm SEM of 2 independent experiments. c , Single channel confocal images of middle right panels merged images presented in Fig. 5d. d , Luciferase assay for the ChIP experiment presented in Fig. 5e. Data are means \pm SEM of 4 independent experiments.

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2. Supplementary Information:

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A. Flat Files

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Item	Present?	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf	A brief, numerical description of file contents. i.e.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
Reporting Summary	Yes	NSMB-A45419-	

		nr-reporting-summary FINAL.pdf
Peer Review Information	Yes	TPRFile_Strubin.pdf

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B. Additional Supplementary Files

8

Type	Number If there are multiple files of the same type this should be the numerical indicator. i.e. "1" for Video 1, "2" for Video 2, etc.	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: <i>Smith_Supplementary_Video_1.mov</i>	Legend or Descriptive Caption Describe the contents of the file
Supplementary Table	1	Supplementary tables.pdf	<i>Supplementary Tables 1-3</i>

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3. Source Data

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Parent Figure or Table	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: <i>Smith_SourceData_Fig1.xls</i> , or <i>Smith_Unmodified_Gels_Fig1.pdf</i>	Data description i.e.: Unprocessed Western Blots and/or gels, Statistical Source Data, etc.
All figures	NSMB-A45419_Source_Data_fullscan.pptx	Unprocessed Western Blots
Figure 1	NSMB-A45419_Figure_1.xlsx	Source Data of graphical representations used in figure 1
Figure 2	NSMB-A45419_Figure_2.xlsx	Source Data of graphical representations used in figure 2
Figure 3	NSMB-A45419_Figure_3.xlsx	Source Data of graphical representations used in figure 3
Figure 4	NSMB-A45419_Figure_4.xlsx	Source Data of graphical representations used in figure 4
Figure 5	NSMB-A45419_Figure_5.xlsx	Source Data of graphical representations used in figure 5
Extended Data Figure 1	NSMB-A45419_Extended_Data_Figure_1.xlsx	Source Data of graphical representations used in extended figure 1
Extended Data Figure 2	NSMB-A45419_Extended_Data_Figure_2.xlsx	Source Data of graphical representations used in extended figure 2
Extended Data Figure 3	NSMB-A45419_Extended_Data_Figure_3.xlsx	Source Data of graphical representations used in extended figure 3
Extended Data Figure 4	NSMB-A45419_Extended_Data_Figure_4.xlsx	Source Data of graphical representations used in extended figure 4
Extended Data Figure 5	NSMB-A45419_Extended_Data_Figure_5.xlsx	Source Data of graphical representations used in extended figure 5
Extended Data Figure 6	NSMB-A45419_Extended_Data_Figure_6.xlsx	Source Data of graphical representations used in extended figure 6
Extended Data Figure 7	NSMB-A45419_Extended_Data_Figure_7.xlsx	Source Data of graphical representations used in extended figure 7

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13 **Smc5/6 silences episomal transcription by a three-step function**

14

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28 **Abstract**

29 In addition to its role in chromosome maintenance, the six-member Smc5/6 complex
30 functions as a restriction factor that binds to and transcriptionally silences viral and other
31 episomal DNA. However, the underlying mechanism is unknown. Here we show that
32 transcriptional silencing by the human Smc5/6 complex is a three-step process. The first step
33 is entrapment of the episomal DNA by a mechanism dependent on Smc5/6 ATPase activity
34 and a function of its Nse4a subunit for which the Nse4b paralog cannot substitute. The second
35 step results in Smc5/6 recruitment to PML nuclear bodies by SLF2 (human ortholog of Nse6).
36 The third step promotes silencing through a mechanism requiring Nse2 but not its SUMO
37 ligase activity. In contrast, the related cohesin and condensin complexes fail to bind to and
38 silence episomal DNA, thus pointing to a property unique to Smc5/6.

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43 **Introduction**

44 The Smc5/6 complex is one of three structurally and functionally related Structural
45 Maintenance of Chromosomes (SMC) complexes that have been identified in eukaryotes.
46 These ring-shaped complexes associate with chromosomal DNA through topological
47 entrapment to play distinct and essential roles in chromosome organization and in maintaining
48 genomic stability ¹. They include condensin, which contains the Smc2 and Smc4 core
49 subunits and mediates chromosome condensation during mitosis, cohesin, which contains
50 Smc1 and Smc3 and holds newly replicated sister chromatids together to ensure their faithful
51 segregation, and the Smc5/6 complex, whose functions remain unclear ¹. As with the other
52 SMC complexes, the backbone of Smc5/6 is formed by a heterodimer of the two Smc
53 proteins, Smc5 and Smc6 (Fig. 1a) ². Each protein contains a central hinge region flanked by
54 two long α -helical arms that fold back on each other forming a coiled-coil structure, thereby
55 bringing the globular N- and C-termini together to form the so-called head domain that has
56 ATPase activity. The two proteins interact via their hinge domain ³, thus forming a V-shaped
57 structure, and associate with four non-SMC proteins (Nse), designated Nse1 to Nse4 ^{4,5}. Nse2
58 has SUMOylation activity and binds the coiled-coil domain of Smc5, whereas Nse4 forms a
59 heterotrimer with Nse1 and Nse3 and bridges the globular head domains of Smc5 and Smc6
60 to form a ring-like structure large enough to encircle two DNA molecules ⁶.

61 The Smc5/6 complex is essential and conserved from yeast to humans and has been attributed
62 a number of functions ⁷⁻⁹. One is to assist repair of double strand-breaks (DSBs) ^{10,11}. Another
63 is to prevent incomplete replication by rescuing stalled replication forks ¹²⁻¹⁴. Both pathways
64 involve homologous recombination between sister chromatids and, if unsuccessful, can lead
65 to genomic instability ^{7,8,15}. Smc5/6 is thought to prevent this by inhibiting formation or
66 promoting removal of toxic recombination intermediates ^{14,16}. Additionally, Smc5/6 has non-
67 repair functions ¹⁷. Specifically, it has been proposed to facilitate replication elongation by

68 binding to and stabilizing sister-chromatid intertwines that accumulate behind the advancing
69 replication fork, thereby relieving the topological tension generated^{18,19}.

70 Recent work revealed that in addition to its essential roles in chromosome maintenance,
71 Smc5/6 also functions as a host restriction factor against hepatitis B virus (HBV)^{20,21}.
72 Specifically, Smc5/6 binds to the circular HBV DNA genome, thereby blocking viral
73 transcription²⁰. HBV antagonizes this restriction by expressing the regulatory HBx protein,
74 which targets Smc5/6 for proteasomal degradation through its recruitment to the cellular
75 DDB1-containing E3 ubiquitin ligase²⁰⁻²². Restriction by Smc5/6 is conserved in mammals²³
76 and is not limited to the HBV genome. Expression of any reporter construct is silenced by
77 Smc5/6, and thus stimulated by HBx expression or by Smc5/6 depletion, regardless of the
78 enhancer and promoter type^{20,24}. However, this occurs only if the DNA remains
79 extrachromosomal. Thus, Smc5/6 functions as a broadly acting restriction factor that operates
80 selectively on extrachromosomal (i.e. episomal) DNA templates to silence HBV and
81 potentially other clinically important DNA viruses^{25,26}. How Smc5/6 detects and binds
82 preferentially to episomal DNA and how it restricts gene transcription remains elusive.

83 As a first step towards addressing these questions, we performed a structure-function analysis
84 of Smc5/6 to identify enzymatic activities and structural properties implicated in its restriction
85 function. Specifically, we tested individual Smc5/6 subunit mutants and/or naturally occurring
86 isoforms for their ability to promote episomal silencing using a functional complementation
87 assay. We also explored whether the other members of the SMC family, cohesin and
88 condensin, exhibit similar restriction activities. Our results reveal that episomal restriction is
89 unique to Smc5/6. They further show that silencing is a three-step process that includes
90 Smc5/6 binding to DNA, recruitment to promyelocytic leukemia nuclear bodies (PML-NBs)
91 by SLF2, and silencing by a mechanism that implicates a novel function of Nse2. They also

92 provide new information about how and under which circumstances the HBx protein interacts
93 with Smc5/6, an issue that may have therapeutic implications.

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96 **Results**97 ***Smc5/6 binds and transcriptionally silences episomal DNA***

98 As a first step towards determining the requirements for Smc5/6 binding to and/or restriction
99 of episomal DNA, we examined whether the other members of the SMC family, cohesin and
100 condensin, would exhibit similar properties (Fig. 1a). We inactivated the three SMC
101 complexes in HepG2 cells using CRISPR/Cas9 and monitored the consequence on episomal
102 gene expression. While depletion of Smc6 led to a strong increase in activity of a transiently
103 transfected luciferase reporter, as expected ²⁰, depletion of Smc2 (condensin) or Smc3
104 (cohesin) had no such effect (Fig. 1b). In line with this finding, siRNA-mediated knockdown
105 of cohesin or condensin also had no effect on viral antigen production from wild-type or
106 HBx-negative HBV in primary human hepatocytes (PHH), in which efficient HBV
107 transcription relies on Smc5/6 degradation by HBx (Extended Data Fig. 1a,b) ^{20,21}. To
108 examine if this lack of effect was due to cohesin and/or condensin failing to bind episomal
109 DNA, we performed chromatin immunoprecipitation (ChIP) using knockout cells
110 complemented with HA-epitope tagged versions of the corresponding SMC subunits. We
111 confirmed that Smc5/6 binds to the episomal reporters (Fig. 1c and Extended Data Fig. 1c) ²⁰.
112 In contrast, cohesin did not bind episomal DNA, despite showing strong binding at
113 chromosomal locations where the complex has been reported to associate ²⁷ (Fig. 1c; see
114 Supplementary Table 3). On the other hand, no signal for condensin was detected at any
115 region examined ²⁸, including episomal DNA, under conditions where condensin complexes
116 containing HA-tagged Smc2 were readily detected (Fig. 1c and Extended Data Fig. 1d).
117 Hence, binding and silencing of episomal DNA templates is unique to Smc5/6 and not a
118 general property of the SMC complexes.

119 ***ATP binding and hydrolysis are required for restriction***

120 Consistent with previous studies ^{20,29}, we found that depletion of any Smc5/6 subunit other
121 than Nse2 results in complex disruption by degradation of the other components, and thus in
122 loss of restriction activity ([Extended Data Fig. 2](#)). We tested individual Smc5/6 subunit
123 mutants and naturally occurring isoforms for both complex formation and functional
124 complementation in a CRISPR/Cas9-knockout background.

125 The N-terminal and C-terminal parts of SMC proteins fold into a globular “head domain” that
126 contains ATP binding and hydrolysis motifs ([Fig. 1a](#)) ³⁰. To examine the role of ATP binding
127 and ATP hydrolysis in Smc5/6-mediated episomal restriction, we engineered Smc5 and Smc6
128 point mutations predicted to abolish either ATP binding or ATP hydrolysis ^{30–32}. The mutants
129 were expressed in HepG2 cells depleted for the corresponding endogenous protein and tested
130 for their ability to restore stable complex formation and restrict transcription of an episomal
131 luciferase reporter construct. Figure 2 shows that depletion of Smc5 resulted in decreased
132 Smc5 and Nse4 protein levels and concomitant increase in reporter gene activity ([Fig. 2a](#),
133 lanes 1 and 6). Complementation with wild-type Smc5 restored normal Nse4 protein levels,
134 indicating stable Smc5/6 complex formation, and reestablished silencing. ([Fig. 2a](#), lanes 6 and
135 7). By contrast, and despite having comparable stabilizing effects on Nse4, the two Smc5
136 ATP binding mutants tested, K86E and D1019A, did not rescue silencing, neither did the
137 ATP hydrolysis mutant E1020A ([Fig. 2a](#), lanes 8-10). The same results were obtained when
138 equivalent mutations were introduced into Smc6 ([Fig. 2b](#)). Thus, both ATP binding and ATP
139 hydrolysis activities of Smc5 and Smc6 are required for episomal silencing.

140 To determine if the lack of restriction activity of the Smc5- and Smc6-mutant complexes
141 reflects a failure to associate with episomal DNA, we performed ChIP experiments. For this,
142 we engineered Smc6-depleted HepG2 cells to express HA-tagged versions of the wild-type or
143 Smc6 mutants. As anticipated, wild-type HA-Smc6 showed strong binding to the episomal
144 luciferase reporter but not to the chromosomal actin gene ([Fig. 2c](#) and [Extended Data Fig. 3a](#))

145 ²⁰. Binding was markedly reduced upon co-expression of HBx, consistent with the HA-tagged
146 Smc6 protein functioning as a subunit of Smc5/6 (Fig. 2c, lanes 2 and 5). By contrast, no
147 signal above background was detected at either site with Smc6 ATP binding mutants, despite
148 them being as effective as wild-type in forming Smc5/6 complexes (Fig. 2c and Extended
149 Data Fig. 3a,b). Although being fully defective for restriction, a Smc5/6 ATP hydrolysis
150 mutant (E1016Q) still associated with the episomal DNA template (Fig. 2d). However, this
151 mutant reproducibly showed lower binding signals compared to wild-type Smc5/6 (Fig. 2d
152 and Extended Data Fig. 3c), suggesting that productive association of Smc5/6 to episomal
153 DNA involves both ATP binding and hydrolysis.

154 Interestingly, HBx degraded wild-type, ATP binding- and ATP hydrolysis-deficient Smc5/6
155 complexes with comparable efficiencies (Extended Data Fig. 3d). This indicates that HBx can
156 bind and target the complex for destruction independently of its ATP-binding status and
157 association to episomal DNA.

158 *Nse4b cannot substitute for Nse4a in episomal silencing*

159 The kleisin subunit Nse4 forms a trimer with Nse1 and Nse3 and connects the globular head
160 domains of Smc5 and Smc6, thus completing the ring-like structure (Fig. 1a)^{4,5}. Mammals
161 have two Nse4 paralogs, Nse4a and Nse4b, which share two highly conserved kleisin
162 domains and show approximately 50% overall identity (Extended Data Fig. 4a)^{29,33,34}. Both
163 proteins can incorporate into a Smc5/6 complex but only Nse4a was recovered in HBx pull-
164 down experiments^{20,29,34}. Consistent with a central role of Nse4 in Smc5/6 formation,
165 CRISPR/Cas9-mediated depletion of Nse4a resulted in degradation of the remaining Smc5/6
166 subunits and consequent loss of episomal silencing (Fig. 3a, lanes 1 and 5). Ectopic
167 expression of Nse4a or Nse4b in this knockout background revealed that the two proteins are
168 equally efficient at supporting assembly of a full Smc5/6 complex (Fig. 3a). However, only
169 the Nse4a-containing Smc5/6 exhibited episomal restriction activity. The Nse4b complex did

170 not (Fig. 3a), and this was due to a failure of the complex to associate with episomal DNA
171 (Fig. 3b and Extended Data Fig. 4b). An Nse4b hybrid protein carrying the N-terminal tail
172 unique to Nse4a was also functionally inactive (Extended Data Fig. 4a,c). Thus, Nse4b can
173 substitute for Nse4a in Smc5/6 but the resulting complex fails to bind to and restrict episomal
174 DNA. As was the case with the ATP-binding and ATP-hydrolysis Smc5/6 mutants, HBx
175 efficiently triggered degradation of the Nse4b-containing Smc5/6 (Fig. 3b). This confirms that
176 HBx-mediated degradation does not require Smc5/6 binding to episomal DNA. Given the
177 relatively low sequence identity between Nse4a and Nse4b, it also suggests that Nse4a does
178 not interact directly with HBx.

179 The Nse4a mRNA can be spliced to produce a shorter isoform of the protein that lacks the C-
180 terminal 104 amino acids implicated in the interaction of Nse4 with Smc5⁵ (Extended Data
181 Fig. 4a). This shorter Nse4a variant stabilized Nse1 and Nse3 but not Smc5 and Smc6,
182 indicating formation of a stable Nse1/Nse3/Nse4 sub-complex (Fig. 3a and Extended Data
183 Fig. 4a)^{34,35}. However, the resulting sub-complex failed to promote restriction (Fig. 3a),
184 suggesting that transcriptional silencing requires a fully assembled complex that includes
185 Smc5 and Smc6.

186 Intriguingly, although ineffective for restriction, Nse4b could substitute, at least partially, for
187 Nse4a in sustaining cell proliferation in a colony formation assay (Fig. 3c)³⁶. This suggests
188 that Nse4a and Nse4b share a common ability to support the essential cellular functions of
189 Smc5/6 and that Nse4a has further acquired an activity uniquely required for Smc5/6 binding
190 to episomal DNA for which Nse4b cannot substitute.

191 *Nse1 and Nse3 DNA-binding mutants are functional in vivo*

192 Smc5/6 loading onto chromosomes is thought to involve the interaction of the Nse1/3/4 sub-
193 complex with DNA, with all three subunits contributing to binding^{37,38}. This is supported by

194 the observation that charge reversal mutations of conserved basic residues in Nse1 and Nse3
195 reduce binding of the Nse1/3/4 sub-complex to DNA *in vitro* and are lethal when introduced
196 into *S. pombe*³⁸. We tested two such mutants, Nse1(K139E) and Nse3(R229E), for episomal
197 silencing using a CRISPR/Cas9-based complementation assay and found that they retain
198 restriction activity (Extended Data Fig. 5). This raises the possibility that Smc5/6 binding to
199 chromosomal or episomal DNA may have different requirements.

200 *Nse2 is implicated in Smc5/6 restriction but not DNA binding*

201 Nse2 is a subunit of Smc5/6 for which no functional equivalent exists in the other SMC
202 complexes. Nse2 binds the coiled-coil domain of Smc5 and possesses SUMO E3-ligase
203 activity that is conserved from yeast to human³⁹⁻⁴¹. Nse2 sumoylates a number of cellular
204 factors involved in diverse chromosome transactions, including components of the replication
205 fork and of cohesin and condensin, as well as subunits of Smc5/6 itself⁴⁰. Although not
206 essential for viability in mice⁴², the SUMO E3 ligase activity of Nse2 has been linked to most
207 Smc5/6 cellular functions^{19,43-46}. Therefore, since SUMOylation has been implicated in
208 transcriptional repression⁴⁷, we investigated the role of Nse2 and its SUMO ligase activity in
209 Smc5/6 episomal restriction. Depletion of Nse2 had little impact on stability of the other
210 Smc5/6 subunits (Fig. 4a and Extended Data Fig. 6a), confirming that Nse2 is dispensable for
211 the integrity of Smc5/6²⁹. Furthermore, the Nse2-depleted complex was degraded by HBx,
212 indicating that Nse2 is not an essential interaction partner of the viral protein (Extended Data
213 Fig. 6a). However, in the absence of Nse2 the complex showed altered silencing activity (Fig.
214 4a and Extended Data Fig. 6a, lanes 1 and 5). Overexpression of Smc5, to which Nse2 binds,
215 did not rescue the Nse2 depletion phenotype and showed weak dominant-negative effects in
216 wild-type cells, presumably due to titration of Nse2 out of the complex. This indicates that
217 Nse2 is important for restriction. Strikingly, however, the Nse2-depleted complex retained its
218 ability to associate with episomal DNA (Fig. 4b and Extended Data Fig. 6b). This points to

219 Nse2 acting at a step subsequent to Smc5/6 loading onto DNA. Furthermore, a SUMO ligase-
220 inactive mutant of Nse2 (C215A) ⁴⁵ was as efficient as the wild-type protein in restoring full
221 restriction activity to the complex in a complementation assay (Fig. 4a and Extended Data
222 Fig. 6a, lanes 5–7). By contrast, the Nse2 mutant was severely compromised in supporting
223 cell growth in a colony formation assay (Fig. 4c). This was unanticipated since the Nse2
224 SUMO ligase activity is generally viewed as non-essential. However, it is consistent with the
225 slow growth phenotype reported for Nse2-depleted human breast cancer cells ⁴⁸. We
226 concluded that Nse2 is important for Smc5/6 restriction while its SUMO ligase activity is not.

227 Previous studies have shown that Smc5/6 localizes to sub-nuclear structures known as
228 promyelocytic leukemia nuclear bodies (PML-NBs) ^{49,50}. This co-localization likely has
229 functional implications with respect to Smc5/6 restriction activity. Indeed, depletion of PML
230 and Sp100, two major constituents of PML-NBs, leads to dispersal of Smc5/6 throughout the
231 nucleus and concomitant loss of episomal restriction activity ⁴⁹. To examine whether Nse2 has
232 a role in the co-localization of Smc5/6 to PML-NBs, we used confocal fluorescence
233 microscopy. Nse2 formed discrete nuclear foci that co-localized with Sp100 foci in the
234 nucleus of PHH (Fig. 4d, upper panels). Similarly, Smc6 co-localized with PML foci, in line
235 with previous studies (Fig. 4d, middle right panels and Extended Data Fig. 6c) ^{49,50}. Depletion
236 of Nse2 by siRNA did not affect the co-localization of Smc6 with PML (Fig. 4d, middle
237 panels, compare siCtrl and siNse2), nor did it change the percentage of cells showing Smc6
238 foci (Fig. 4d, lower panels). Thus, Nse2 is not implicated in Smc5/6 recruitment to PML-NBs.

239 Collectively, these data indicate that restriction by Smc5/6 involves a SUMO ligase-
240 independent function of Nse2 that operates at a step other than episomal DNA binding or
241 localization to PML-NBs.

242 ***SLF2 promotes silencing by recruiting Smc5/6 to PML-NBs***

243 In addition to Nse1–Nse4, the yeast Smc5/6 contains two less well-characterized non-SMC
244 subunits named Nse5 and Nse6⁵¹. These two proteins form a dimer and associate loosely with
245 the hexameric core Smc5/6 complex through contacts with both Smc5 and Smc6⁵². The
246 distantly related SLF1 and SLF2 proteins are believed to be the functional human orthologs of
247 yeast Nse5 and Nse6⁵³. Little is known about how the Nse5/6 dimer contributes to Smc5/6
248 function. In yeast, Nse5/6 appears to play a key role in the recruitment and loading of Smc5/6
249 onto chromatin, in particular to sites of DSBs and at stalled replication forks^{54–56}, as well as
250 other chromosomal regions⁵⁷. Similarly, the recruitment of human Smc5/6 to sites of DNA
251 damage has been shown to require the Nse6 ortholog SLF2 and its interaction with SLF1⁵³.
252 To determine whether SLF1 and SLF2 play a role in Smc5/6 restriction, we individually
253 depleted these proteins. Depletion of SLF1 had no effect on the levels of SLF2 and the core
254 Smc5/6 subunits, nor did it alter Smc5/6 binding and silencing of an episomal luciferase
255 reporter (Fig. 5a,b. Depletion of SLF2 also had no effect on Smc5/6 protein stability (Fig. 5a,
256 lanes 1 and 5). However, SLF1 protein levels were reduced, suggesting that SLF1 is stable
257 only in association with SLF2. Strikingly, SLF2-depleted cells showed markedly increased
258 episomal reporter gene activity, indicating loss of silencing (Fig. 5a and Extended Data Fig.
259 7a, lanes 1 and 5). Moreover, HBx normally degraded Smc5/6 in these cells, arguing against
260 SLF1 or SLF2 being direct interaction partners of HBx, and this had little further effect on
261 transcription of the episomal reporter, consistent with SLF2 acting through the Smc5/6
262 pathway (Extended Data Fig. 7a). Collectively, these data indicate that SLF2 plays an SLF1-
263 independent role in Smc5/6 restriction activity.

264 Unexpectedly, ChIP analysis revealed that Smc5/6 still associated with episomal DNA in the
265 absence of SLF2 (Fig. 5b and Extended Data Fig. 7b). Moreover, loss of SLF2 did not impact
266 on cell viability (Fig. 5c). We therefore evaluated whether SLF2 influences Smc5/6 cellular
267 localization. Fluorescence microscopy studies revealed that SLF2 co-localizes with Smc6 in

268 PML-NBs in PHH (Fig. 5d, upper panels), and that PML-NBs form normally in the absence
269 of SLF2 (Fig. 5d, middle panels). Remarkably, however, depletion of SLF2 led to
270 disappearance of Smc5/6 from PML-NBs (Fig. 5d, middle right panels and Extended Data
271 Fig. 7c). Furthermore, binding of HA-PML to episomal DNA requires the SLF2 subunit of
272 Smc5/6, consistent with the notion that one function of SLF2 is to recruit Smc5/6 and hence
273 episomal DNA to PML-NBs for silencing (Fig. 5e and Extended Data Fig. 7d). Thus, SLF2 is
274 essential for Smc5/6 restriction but, in contrast to its role as a DNA-loading factor⁵³, SLF2
275 acts independently of SLF1 to recruit Smc5/6 to PML-NBs.

276

277 **Discussion**278 ***Only Smc5/6 binds to and restricts episomal DNA***

279 In addition to its essential roles in DNA replication and repair, Smc5/6 also functions as a
280 restriction factor against HBV. Smc5/6 binds to and silences the episomal HBV genome and,
281 more generally, any extrachromosomal reporter template^{20,21,24}. In this study, we explored the
282 requirements for Smc5/6 to perform its restriction function. A major finding is that binding to
283 and silencing of episomal DNA is unique to Smc5/6 and not a general property of the SMC
284 complexes. This is consistent with previous observations that HBx targets only Smc5/6 for
285 destruction²¹. It is also in agreement with our observations that knockdown of Smc5/6 but not
286 of cohesin or condensin rescues an HBx-deficient HBV in PHH ([Extended Data Fig. 1a,b](#)).
287 Thus, Smc5/6 recognizes a feature of episomal DNA templates not detected by the other two
288 SMC complexes.

289 ***Role of Smc5/6 ATPase activity in episomal DNA binding***

290 We found that Smc5/6 complexes containing Smc5 or Smc6 subunits defective in ATP
291 binding or hydrolysis all failed to restrict episomal gene expression. However, whereas ATP
292 binding mutants were unable to associate with DNA, consistent with studies in yeast⁶, the
293 Smc6 ATP hydrolysis mutant we tested, E1016Q, retained DNA-binding ability. *In vitro*
294 studies suggest that Smc5/6 associates with DNA in a two-step process^{6,38,58}. The first step
295 requires ATP binding by Smc5 and Smc6 and involves electrostatic interactions of the
296 complex with the DNA. The second step depends on ATP hydrolysis and leads to the
297 topological entrapment of the DNA within the ring-shaped Smc5/6. A similar mechanism has
298 been proposed for the loading of prokaryotic SMC complexes⁵⁹ and of cohesin⁶⁰ onto
299 chromosomes. Interestingly, the Smc6 ATP hydrolysis mutant we tested showed reduced
300 binding to episomal DNA templates compared to wild type ([Fig. 2d](#)). This suggests that the

301 hydrolysis mutant complex binds but fails to entrap DNA in its ring structure. However, we
302 cannot exclude that ATP hydrolysis plays a role subsequent to Smc5/6 DNA binding (see
303 below). Nevertheless, these findings provide further evidence that Smc5/6 functions through
304 its binding to the episomal DNA template, and suggest that an ATPase-dependent topological
305 entrapment of the DNA is a prerequisite for silencing.

306 ***Role of Nse4 in Smc5/6 binding to episomal DNA***

307 Consistent with previous studies ^{4,29}, we found that Nse4b could physically substitute for
308 Nse4a in the Smc5/6 complex. However, the resulting complex was defective for interaction
309 with episomal DNA templates (Fig. 3). This differs from the situation with the Smc5/6 ATP-
310 hydrolysis mutant, which retained some DNA binding activity, suggesting that Nse4a has
311 essential roles at an early step that precedes entrapment of the DNA and for which Nse4b
312 cannot substitute. Our results may explain previous observations showing that, when
313 overexpressed together with Nse3, Nse4b but not Nse4a stimulated expression of a transiently
314 transfected reporter, and this was dependent on the Nse3-Nse4b interaction ³⁴. Presumably,
315 overexpressed exogenous Nse4b competes Nse4a out of the complex, thereby preventing
316 binding to and silencing of the episomal reporter. Interestingly, despite being fully defective
317 for episomal silencing, the Nse4b complex was partially competent at supporting normal cell
318 growth. This raises the possibility that Smc5/6 binding to episomal versus chromosomal sites
319 occurs by different mechanisms. It will be interesting to examine if purified Nse4a- and
320 Nse4b-containing Smc5/6 exhibit different DNA substrate preferences *in vitro* ⁶¹.

321 Intriguingly, while Nse4a is expressed in most human tissues ⁶², expression of Nse4b is
322 restricted to testis ⁶³, and evidence has been presented that in testis Smc5/6 contains Nse4b
323 rather than Nse4a ³⁴. This raises the question of whether there is any requirement for the testes
324 to express a Smc5/6 devoid of episomal silencing activity.

325 *Nse2 and SLF2 function downstream of Smc5/6 binding*

326 Whereas Smc5, Smc6 and Nse4a all play a key role in Smc5/6 core integrity and binding to
327 episomal DNA, Nse2 and the SLF1/2 (human Nse5/6) sub-complex are dispensable.
328 Nonetheless, Nse2 and SLF2, but not SLF1, are essential for Smc5/6 restriction function. This
329 points to an involvement of Nse2 and SLF2 at a step following Smc5/6 binding to episomal
330 DNA. Indeed, we show that SLF2 is implicated in the co-localization of Smc5/6 to PML-
331 NBs, which are essential for Smc5/6-mediated episomal restriction ⁴⁹. Since Smc5/6 binds
332 normally to episomal DNA in the absence of SLF2 (Fig. 5b), we conclude that a major
333 function of SLF2 is to recruit Smc5/6 and, possibly the associated episomal DNA, to PML-
334 NBs. As this occurs independently of SLF1, the process must involve a pathway distinct from
335 that operating at sites of DNA damage, where the recruitment by SLF2 of human Smc5/6
336 requires its interaction with SLF1 ⁵³. These findings further implicate PML-NBs in restriction
337 and identify a new role for SLF2 in Smc5/6 activity.

338 Interestingly, a role for SLF2 and Smc5/6 in mediating repression of unintegrated, circular
339 HIV DNA species has recently been reported ²⁵. In agreement with our findings, it was shown
340 that SLF1 was not required for SLF2 restriction function. However, in contrast with our data
341 suggesting that SLF2 acts subsequent to Smc5/6 binding to DNA, this study implicates SLF2
342 in the association of Smc5/6 with the unintegrated viral DNA. We currently have no
343 explanation for the discrepancy. Evidence has been presented that Smc5/6 has similar
344 restriction activity in diverse mammalian cell lines ²³. This makes it unlikely that the
345 inconsistency between the two studies reflects a cell-type specific difference in SLF2
346 function. Differences in the nature of the episomal templates are also unlikely, as similar
347 unintegrated lentiviral reporter constructs were used in both studies. We note that the two
348 studies measured Smc5/6 binding by ChIP using different HA-tagged subunits (HA-Nse2 by
349 Dupont et al. versus HA-Smc6 in our study). How this might account for the discrepancy

350 remains unclear. Perhaps the HA tag on Nse2 becomes inaccessible or the protein dissociates
351 from the complex in the absence of SLF2.

352 Nse2, on the other hand, is required neither for Smc5/6 binding to episomal DNA or for its
353 co-localization to PML-NBs (Fig. 4). This points to a role for Nse2 subsequent to Smc5/6
354 recruitment to PML-NBs, thus raising the possibility that Nse2 participates directly in
355 transcription silencing in the context of PML-NBs. Surprisingly, we found that Nse2 performs
356 this function independently of its SUMO E3 ligase activity. This was unexpected, given both
357 the fact that the Nse2 E3 ligase plays a role in most of the other Smc5/6 activities³⁹⁻⁴¹ and the
358 importance of SUMOylation in PML-NB functions⁶⁴. This leads us to conclude that Nse2
359 contributes to Smc5/6 restriction by performing an unknown function required for silencing
360 that remains to be determined. SMC complexes are dynamic machines able to adopt distinct
361 folded configurations and can undergo conformational changes to perform their functions⁶⁵⁻
362⁶⁷. We speculate that Nse2 may act as an ancillary effector of the Smc5/6 as Nse2 is not
363 essential for the integrity of the complex or for binding to episomal DNA. Nse2 possibly
364 influences Smc5/6 complex interactions with DNA to trigger the silencing function. Notably,
365 Nse2 binds a positively-charged patch on the Smc5 elbow, a region also thought to interact
366 with DNA already loaded into the complex, suggesting interplay between Nse2, Smc5, and
367 the loaded DNA³⁹. Furthermore, Nse2 has been reported to possess two SUMO-Interacting
368 Motif (SIM)-like motifs⁶⁸. Nse2 may be recruited to SUMO-conjugated PML through its
369 SIM sequences. Further work is necessary to shed light on these important issues.

370

371 ***Smc5/6 degradation by HBx***

372 Biochemical and structural studies have firmly established that HBx binds to DDB1 to recruit
373 Smc5/6 to the DDB1-containing E3 ligase and induce its degradation^{20,22}. However, how

374 HBx interacts with the Smc5/6 complex remains unknown and is of potential therapeutic
375 interest. Single subunit overexpression experiments and yeast two-hybrid interaction assays
376 failed to identify an HBx-interacting partner, suggesting that stable interaction involves more
377 than one Smc5/6 subunit (M.S., unpublished data). We show here that HBx retains its ability
378 to trigger Smc5/6 degradation in the absence of Nse2 or SLF2, or when Nse4b replaces Nse4a
379 in the complex. This argues against these proteins being key ubiquitin substrates or mediating
380 Smc5/6 interaction with HBx. Since SLF2 is responsible for the recruitment of Smc5/6 to
381 PML-NBs, the results also point to HBx triggering degradation of Smc5/6 independently of
382 its co-localization to PML-NBs. HBx efficiently degrades both ATP binding and ATP
383 hydrolysis mutant Smc5/6 complexes. Since Smc5/6 containing Nse4b or defective in ATP
384 binding fail to associate with DNA, we conclude that HBx binds to and triggers degradation
385 of Smc5/6 independently of DNA. Furthermore, Smc5/6, like condensin ⁶⁷ and cohesin ⁶⁶, is
386 predicted to adopt distinct conformations during its ATP hydrolysis cycle ^{4,35,37,58,69}. Our
387 results therefore also point to HBx binding to Smc5/6 regardless of its ATP-dependent
388 conformational state.

389 ***A three-step mechanism of restriction***

390 The exact mechanism whereby Smc5/6 represses episomal gene transcription and the precise
391 nature of the DNA structure recognized by Smc5/6 remain to be determined. It is possible that
392 the binding of Smc5/6 to episomal DNA is topological. Indeed, *in vitro*, the yeast and the
393 human complexes detect and bind DNA tertiary structures displaying crossed DNA helices
394 such as plectonemes and catenated DNA templates using the energy of ATP hydrolysis to
395 promote their compaction^{6,61}. Whether the same is true *in vivo* in a cellular context remains to
396 be resolved. The purified human complex used in these studies lacks the Nse2 and SLF2
397 subunits ⁶¹, which we found play key roles in restriction. Furthermore, yeast Smc5/6 shows
398 similar compaction and plasmid catenation activities *in vitro* ^{6,58}, yet does not appear to

399 silence episomal DNA templates *in vivo* (M.S., unpublished data). Thus, although ATP
400 hydrolysis is required for restriction (Fig. 2), it is unlikely that Smc5/6 silences episomal gene
401 expression solely by an ATPase-dependent DNA entrapment and/or compaction mechanism.

402 Rather, our findings that Nse2 and SLF2 have distinct roles and act after Smc5/6 binding to
403 episomal DNA would lead us to propose the following three-step silencing mechanism. The
404 first step involves Smc5/6 binding to and, likely, topological entrapment of the episomal
405 DNA template. The second leads to recruitment of Smc5/6 to PML-NBs by SLF2 through a
406 pathway independent of SLF1. The third results in episomal silencing by a mechanism that
407 implicates an Nse2 function other than its SUMO E3 ligase activity. PML-NBs are well
408 known to serve a host antiviral defense function by associating with the genomes of several
409 DNA viruses soon after their delivery into the nucleus to inhibit their expression⁷⁰. It will be
410 interesting to determine whether cells use the same antiviral mechanism to target DNA
411 viruses other than HBV.

412

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420 Author Contributions

421 F.A., R.K.B., S.P.F. and M.S. conceived the study and designed the experiments. D.R.
422 performed the PHH experiments shown in Extended Data Fig. 1a,b. D.R. and D.K. performed
423 the confocal microscopy experiments shown in Fig. 4d. R.K.B. and D.K. performed the
424 confocal microscopy experiments shown in Fig. 5d. F.A. performed all the other experiments.
425 B.B. contributed to several figures. AD contributed to the revision of the manuscript. All
426 authors interpreted the results. F.A. and M.S. wrote the manuscript with input from all
427 authors.

428 Competing interests

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431

432 **Figure Legends**433 **Fig. 1 | Only Smc5/6, not cohesin or condensin, binds to and silences episomal templates.**

434 **a**, Schematic diagram illustrating the similar ring-shaped organization of the three SMC
435 complexes, condensin, cohesin and Smc5/6. See text for details. CAP-H is the kleisin
436 counterpart of Nse4 in the Smc2/4 condensin complex ⁹.

437 **b**, HepG2 cells were transduced with lentiviral constructs expressing Cas9 alone (Ctrl) or
438 Cas9 together with single guide RNAs (sgRNA) targeting the indicated Smc subunits. After
439 selection, cells were transfected in parallel with a luciferase reporter plasmid. Luciferase
440 assay and Western blot analysis were performed 5 days later. Luciferase activity is relative to
441 that measured in control cells, which was set to 1. Data are means \pm SEM of 5 independent
442 experiments. Statistical significance relative to the control (Ctrl) was calculated by one-way
443 ANOVA with Dunnett's multiple comparison correction; * $p=0.014$, *** $p=0.0008$, ns: not
444 significant ($p>0.05$).

445 **c**, Cells depleted as in **b** and complemented with the corresponding HA-epitope tagged Smc
446 subunit were co-transduced with an integrase-defective lentiviral vector to deliver the
447 luciferase reporter as an extrachromosomal DNA ⁷², and with lentiviruses encoding GFP or
448 GFP-HBx. Binding of the proteins at the indicated regions was monitored 4 days later by
449 ChIP using anti-HA antibodies. Values are expressed as percentage of input DNA recovered.
450 Data are means \pm SEM of 2 independent experiments. Statistical significance for each region
451 (actin, cohesin or luciferase) relative to HA-Smc2/GFP transduced cells was calculated with
452 by one-way ANOVA with Dunnett's multiple comparison correction. Only p -values <0.05 are
453 labeled; ** $p<0.01$. Note that in these and subsequent experiments, the complementing HA-
454 Smc6 is typically overexpressed and only the minor fraction assembled into a Smc5/6
455 complex binds to DNA and is degraded by HBx ²⁰.

456 **Fig. 2 | Smc5 and Smc6 ATP binding and hydrolysis are required for episomal DNA**
457 **restriction.**

458 **a**, HepG2 cells depleted of Smc5 (grey bars) or not (black bars) were generated as before.
459 Cells were transfected with a luciferase reporter plasmid, and then transduced with
460 lentiviruses encoding GFP, wild-type Smc5 or the indicated Smc5 ATP binding or ATP
461 hydrolysis mutants. Luciferase assay and western blot analysis were performed 5 days later.
462 Luciferase values are relative to those in non-depleted cells expressing GFP. Data are means \pm
463 SEM of 3 independent experiments. Statistical significance relative to matched GFP control
464 was calculated by one-way ANOVA with Dunnett's multiple comparison correction. Only p-
465 values <0.05 are labeled; * $p=0.0211$, *** $p=0.0005$.

466 **b**, Similar experiments as in **a** were performed to test Smc6 derivatives carrying equivalent
467 mutations for functional complementation of Smc6 CRISPR knockout cells. Data are means \pm
468 SEM of 5 independent experiments. Statistical significance relative to matched GFP control
469 was calculated by one-way ANOVA with Dunnett's multiple comparison correction. Only p-
470 values <0.05 are labeled; * $p=0.0295$, ** $p=0.0069$.

471 **c**, HepG2 cells depleted of Smc6 as in **b** and expressing HA-tagged versions of GFP, wild-
472 type or the indicated ATP-binding mutants of Smc6 were co-transduced with an integrase-
473 defective lentiviral luciferase reporter construct together with lentiviruses encoding GFP or
474 GFP-HBx. Anti-HA ChIP and Western blot analysis were performed 5-6 days later as in Fig.
475 1b. Data are means \pm SEM of 3 independent experiments. Unessential lanes were removed
476 from the original blot images. Statistical significance for luciferase reporter region relative to
477 HA-GFP/GFP control was calculated by one-way ANOVA with Dunnett's multiple
478 comparison correction; † $p=0.056$, and all other comparisons were $p>0.05$.

479 **d**, Same as **c** but with cells expressing HA-tagged wild type or the indicated Smc6 ATP
480 hydrolysis mutant. Data are expressed as means \pm SEM of 3 independent experiments.
481 Statistical significance for luciferase reporter region relative to HA-GFP/GFP control was
482 calculated by one-way ANOVA with Dunnett's multiple comparison correction; Only p-
483 values <0.05 are labeled; *p= 0.0258, **p= 0.0024.

484

485 **Fig. 3 | Nse4b-containing Smc5/6 complexes support cell viability but fail to silence**
486 **episomal DNA.**

487 **a**, HepG2 cells depleted of Nse4a (grey bars) or not (black bars) were transfected with a
488 luciferase reporter plasmid, and shortly after transduced with lentiviruses encoding GFP,
489 Nse4a (long), a shorter splicing isoform of Nse4a (short), or Nse4b as indicated. Luciferase
490 assay and Western blot analysis were performed and are presented as in Fig. 2a. Expression of
491 Nse4b was inferred from its stabilization effect on the other Smc5/6 subunits (lane 8). Data
492 are means \pm SEM of 4 independent experiments. Statistical significance relative to matched
493 GFP control was calculated by one-way ANOVA with Dunnett's multiple comparison
494 correction. Only p-values <0.05 are labeled; *p= 0.0120.

495 **b**, Control HepG2 cells expressing HA-GFP, or Nse4a-depleted cells expressing HA-Nse4b
496 or HA-Nse4a, were co-transduced with an integrase-defective lentiviral luciferase reporter
497 construct and with lentiviruses encoding GFP or GFP-HBx. Anti-HA ChIP and Western blot
498 analyses were performed 5 days later as before. Data are means \pm SEM of 2 independent
499 experiments. Statistical significance for luciferase reporter region relative to HA-GFP/GFP
500 control was calculated by one-way ANOVA with Dunnett's multiple comparison correction.
501 Only p-values <0.05 are labeled; *p= 0.0455, ****p <0.0001 .

502 **c**, Colony formation assay. Control HepG2 cells and cells depleted of Nse4a were either
503 mock-transduced or transduced with lentiviral constructs expressing the indicated proteins.
504 Cells were then seeded at low density and grown under selective conditions for 14-21 days
505 prior to fixation and staining with crystal violet. Shown is one representative experiment out
506 of three performed.

507

508 **Fig. 4 | Nse2 but not its SUMO ligase is essential for episomal restriction but not for**
509 **Smc5/6 DNA binding and co-localization with PML bodies.**

510 **a**, Control HepG2 cells (black bars) and cells depleted of Nse2 (grey bars) were transfected
511 with a luciferase reporter plasmid, and subsequently transduced with lentiviruses encoding
512 GFP, Flag-tagged wild-type Nse2 (F-Nse2) or a Nse2-SUMO E3 ligase-defective mutant
513 (C215A)⁴⁵, or Smc5. Luciferase assay and Western blot analysis were performed as before.
514 Luciferase values are relative to GFP in control cells. Data are means \pm SEM of 4 independent
515 experiments. Statistical significance relative to matched GFP control was calculated by one-
516 way ANOVA with Dunnett's multiple comparison correction. Only p-values <0.05 are
517 labeled; **p <0.01 , ***p= 0.0005.

518 **b**, HepG2 cells depleted of Smc6 and expressing HA-tagged Smc6 were transduced with
519 lentiviral constructs expressing Cas9 alone (ctrl) or Cas9 together with a sgRNA targeting
520 Nse2. Cells were then co-transduced with an integrase-defective lentiviral luciferase reporter
521 construct and with lentiviruses encoding GFP, GFP-HBx or Flag-tagged Nse2. Anti-HA ChIP
522 and Western blot analysis were performed 5 days later as before. Data are means \pm SEM of 3
523 independent experiments. Statistical significance for luciferase reporter region relative to GFP
524 control (Ctrl) was calculated by one-way ANOVA with Dunnett's multiple comparison
525 correction. Only p-values <0.05 are labeled; ****p <0.0001 .

526

527 **c**, Colony formation assay of control HepG2 cells or cells depleted of Nse2 and expressing the
528 indicated proteins from lentiviral vectors. Shown is one representative experiment out of three
529 performed.

530 **d**, Confocal images of PHH transfected with either a non-targeting control siRNA (siCtrl) or
531 with siRNA against Nse2 (siNse2) and incubated for 13 days. Cells were stained for Sp100 or
532 Smc6 (green) and for Nse2 or PML (red) as indicated. The boundaries of nuclei are outlined
533 in white circles based on DAPI signal (not shown). The bottom graphs reflect the percentage
534 of nuclei without (left) or with (right) Smc6 foci. The data represent the mean \pm SD of 2
535 independent experiments, with 328-428 nuclei analyzed per siRNA treatment in each
536 experiment. Statistical significance relative to siCtrl was calculated by paired t-test; ns: not
537 significant ($p > 0.05$).

538

539 **Fig. 5 | SLF2 promotes silencing by recruiting Smc5/6 to PML-NBs**

540 **a**, HepG2 cells were transduced with lentiviral constructs expressing Cas9 alone (ctrl), or
541 Cas9 together with sgRNA as indicated. Cells were transfected with a luciferase reporter
542 plasmid and transduced with GFP or GFP-HBx. Analyses were performed as before. Data are
543 means \pm SEM of 3 independent experiments. Statistical significance relative to Ctrl was
544 calculated by one-way ANOVA with Dunnett's multiple comparison correction. Only p-
545 values < 0.05 are labeled; *** $p < 0.001$.

546 **b**, Smc6-depleted HepG2 cells expressing HA-tagged Smc6 were transduced with constructs
547 expressing Cas9 alone (Ctrl), or Cas9 together with sgRNA as indicated. Cells were co-
548 transduced with an integrase-defective luciferase lentivirus and with the indicated
549 lentiviruses. Analyses were as before. Data are mean \pm SEM of 2 independent experiments.

550 Statistical significance for luciferase region relative to Ctrl-GFP was calculated by one-way
551 ANOVA with Dunnett's multiple comparison correction. Only p-values <0.05 are labeled;
552 **p= 0.0042.

553 **c**, Colony formation assay of control HepG2 cells or cells depleted of SLF2 and transduced
554 with lentiviruses encoding either GFP or SLF2.

555 **d**, Confocal images of PHH transfected with either a control siRNA (siCtrl) or with siRNA
556 against SLF2 (siSLF2) and incubated for 13 days. Cells were stained for Sp100 or Smc6
557 (green) and for SLF2 or PML (red) as indicated. Data are presented as in Fig. 4d and are mean
558 \pm SD of 3 independent experiments, with 56-119 nuclei analyzed per siRNA treatment in each
559 experiment. Statistical significance relative to siCtrl was calculated by paired t-test; *p=
560 0.0156, **p= 0.0027.

561 **e**, HepG2 cells expressing HA-PML-1 were transduced with lentiviral constructs expressing
562 Cas9 alone (Ctrl), or Cas9 together with sgRNA targeting SLF2. Cells were co-transduced
563 with an integrase-defective luciferase lentivirus and with lentiviruses expressing GFP, ICP0 (a
564 protein from herpes simplex virus type 1 known to induces PML degradation ⁷³), SLF2 or
565 HBx. Analyses were as before. Data are mean \pm SEM of 4 independent experiments with n=4
566 for all conditions except for HBx n=3. Statistical significance for luciferase region relative to
567 Ctrl-GFP was calculated by one-way ANOVA with Dunnett's multiple comparison
568 correction. Only p-values <0.05 are labeled; *p<0.05, **p<0.01, ***p<0.001.

569

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- 725

726 **Methods**727 *Cell Lines and Culture Conditions*

728 The cell lines used in this study were the human embryonic kidney (HEK) cell line HEK
729 293T/17 (ATCC CRL-11268), the human hepatoma cell line HepG2 (ATCC HB-8065) and
730 HeLa. All cell lines and their derivatives were grown at 37°C under 5% CO₂ in Dulbecco
731 modified Eagle medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal calf serum
732 (Gibco), 1% (v/v) penicillin/streptomycin solution, 2 mM l-glutamine, 1 mM sodium
733 pyruvate, and 0.1 mM MEM non-essential amino acids solution (all from Life Technologies).

734 *Primary Human Hepatocytes*

735 Primary human hepatocytes (PHH) isolated from deceased donor livers were purchased from
736 BioIVT (Westbury, NY) and maintained in William's E medium with added supplements as
737 specified by the vendor. Consent was obtained from the donor or the donor's legal next of kin
738 for use of the samples and their derivatives for research purposes using institutional review
739 board (IRB)-approved authorizations.

740 *Expression Plasmids, Complementing Constructs and Smc5/6 Subunit Mutants*

741 GFP and GFP-HBx were expressed from the lentiviral vector pWPT (Addgene #12255) as
742 described²⁰. All wild-type and mutant Smc5/6 complex subunits and naturally occurring
743 isoforms thereof were produced by cloning human codon-optimized, chemically synthesized
744 (System Biosciences) gene sequences into pCDH-CMV-MCS-EF1 α -copGFP lentiviral vector
745 (System Biosciences #CD511B) under control of the CMV promoter. Complementing
746 constructs resistant to CRISPR/Cas9 cleavage were generated by inserting silent mutations in
747 the region of the mRNA covered by the sgRNA. The Smc5(K86E) and Smc6(K82E) point
748 mutations in the Walker A nucleotide-binding motif, and the Smc5(D1019A) and
749 Smc6(D1015A) mutations in the Walker B motif, are predicted to abolish ATP binding³¹.

750 The Smc5(E1020A), Smc6(E1016A) and Smc6(E1016Q) substitutions in the Walker B motif
751 are predicted to prevent hydrolysis of the bound ATP³⁰⁻³². DNA sequences of the mutants are
752 available upon request. The secreted Gaussia luciferase (GLuc) used as reporter was
753 expressed from pCMV-GLuc (New England BioLabs) for transient transfection and from
754 pCDH-CMV-Gluc-EF1 α -copGFP when delivered as episomal DNA using an integrase-
755 defective (D116A) lentiviral vector.

756 Gene knockout was performed using the puromycin-resistant CRISPR-Cas9 lentiviral vector
757 plentiCRISPR v2 (Addgene plasmid #52961) encoding Cas9 and containing two BsmBI
758 cloning sites for insertion of single-guide RNA (sgRNA)⁷¹. The sgRNA sequences specific to
759 the genes of interest were selected using the online CRISPR-Cas9 Design Tool hosted by the
760 Feng Zhang Laboratory (<http://crispr.mit.edu>), Chopchop (<http://chopchop.cbu.uib.no/>) or
761 CRISPOR (<http://crispor.tefor.net/>), and cloned as annealed oligonucleotides into
762 plentiCRISPR v2 as described⁷¹. The sgRNA sequences used in this study are listed in
763 Supplementary Table 1.

764 ***Lentivirus Production and Titration***

765 VSV-G pseudotyped recombinant lentiviruses were produced by transient transfection of
766 HEK 293T/17 cells using the calcium phosphate method. Briefly, 4.5x10⁶ cells were plated in
767 a 10-cm dish and transfected 16 h later with 15 μ g lentiviral vector plasmid, 10 μ g of
768 packaging plasmid psPAX2 (Addgene plasmid #12260), and 5 μ g of envelope plasmid
769 pMD2G (Addgene plasmid #12259). For production of integrase-defective lentiviruses,
770 psPAX2 was replaced by p8.9NdSB (gift from Jeremy Luban) encoding a catalytically
771 inactive integrase point mutant (D116A). The culture medium was changed 8 h post-
772 transfection. Viral containing supernatants were collected 48 h later, filtered through PVDF
773 0.45 μ m filters (Merck-Millipore) and stored at -80°C. The titer of lentiviruses expressing

774 GFP was estimated by infection of HeLa cells with serially diluted viral supernatants and
775 FACS analysis 4 days later for GFP-positive cells.

776 ***Transfection, Transduction, CRISPR-Cas9 Knockout and Complementation Assay***

777 HepG2 cells were typically seeded at a density of $3-6 \times 10^5$ cells per well of a 6-well dish and
778 transduced the next day with sgRNA-expressing lentiviruses or with the empty parental
779 plentiCRISPR v2 as indicated in the figures. After five days of culture, transduced cells were
780 selected with 5 $\mu\text{g}/\text{mL}$ puromycin (Invivogen) for 24 h. Cells were then trypsinized, counted,
781 replated in 6-well dishes at a density of $3-6 \times 10^5$ cells per well, and immediately reverse-
782 transfected with 15-30 ng of the luciferase reporter plasmid pCMV-GLuc and 1-2 μg of
783 empty EBS-PL vector ²⁰. Transfection was performed using X-tremeGENE HP DNA
784 transfection reagent (Roche) or polyethylenimine (PEI; Polysciences #23966) following the
785 manufacturer's instructions. Where indicated, cells were then transduced 2 h later with control
786 or complementing lentiviral constructs, and/or with GFP or GFP-HBx. For ChIP and pull
787 down experiments, cells were first transduced with HA-tagged versions of the proteins of
788 interest and 5 days later with sgRNAs targeting the corresponding endogenous protein.
789 Experiments with HA-tagged Smc6 were either performed as described above (Fig. 1c and
790 [Extended Data Figs. 1c,d and 2](#)), or starting with a CRISPR/Cas9-edited HepG2 cell clone
791 (6.1.2) isolated following standard methods and expressing low levels of endogenous Smc6
792 leading to loss of restriction activity but normal cell growth. After expansion under
793 puromycin selection, $2-5 \times 10^6$ cells were seeded onto one 10- to 15-cm culture dish and
794 transduced the following day with luciferase reporter gene GLuc delivered as an episomal
795 DNA using an integrase-defective (D116A) lentivirus, and with GFP or GFP-HBx as
796 indicated.

797 ***Luciferase Reporter Gene Assay***

798 Luciferase activities were measured 4-6 days after reporter transfection or transduction. Five
799 μL of culture supernatants were mixed with 50 μL of GLuc assay buffer (100 mM NaCl, 35
800 mM EDTA, 0.1% Tween 20, 300 mM sodium ascorbate, 4 μM coelenterazine in 1X
801 phosphate-buffered saline [PBS]) and luminescence was immediately measured in triplicates
802 in a 96 microplate luminometer (Glomax; Promega).

803 ***Western Blotting***

804 Cells were lysed in 2% SDS in water, and the lysates were briefly sonicated and denatured at
805 95°C for 5 minutes. Protein concentrations were estimated using the PierceTM BCA Protein
806 Assay kit (Thermo Scientific). Proteins (20 μg) were separated on 8-18% SDS-PAGE
807 gradient gels and electroblotted onto nitrocellulose membranes. The membranes were blocked
808 in 5% (w/v) milk – TBST 0.1% [TBS buffer (10 mM Tris-HCl (pH 7.5), 500 mM NaCl)
809 supplemented with 0.1% Tween-20] and probed with primary antibodies overnight at 4°C in
810 5% milk – TBST 0.1%. The membranes were then washed thrice with TBST 0.1% for 10
811 min, and incubated with secondary antibodies for 1 h at RT. The membranes were finally
812 washed thrice with TBST 0.1% for 10 min. Detection was performed with SuperSignal West
813 Pico PLUS chemiluminiscent substrate (Thermo Scientific, 34580) according to the
814 manufacturer's protocol. Images were acquired with the ChemicDoc XRS luminescence
815 imager from Bio-Rad. The primary and secondary antibodies used are listed in Supplementary
816 Table 2.

817 ***ChIP and Quantitative PCR***

818 For ChIP experiments, the episomal DNA template was delivered using an integrase-defective
819 lentiviral construct to increase the fraction of cells containing an episomal template and limit
820 the copy number per cell. ChIP analysis was performed using chromatin extracted from about
821 $4\text{-}10 \times 10^6$ HA-tagged protein-expressing HepG2 cells cultured in a 10-15 cm diameter dish.

822 Cells were harvested with trypsin-EDTA and collected by low-speed centrifugation. Cells
823 were rinsed twice with ice-cold PBS, resuspended and fixed for 45 min with the protein-
824 protein crosslinker ChIP Cross-link Gold (Diagenode) following the manufacturer's
825 instructions, rinsed again twice with ice-cold PBS, and further fixed with 1% formaldehyde
826 (Sigma Aldrich) for 10 min at RT before quenching with 330 mM glycine. After two further
827 washes with ice-cold PBS, cells were resuspended and lysed for 10 min at 4°C in 1 mL
828 Nuclear Extraction buffer (10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.5% NP-40)
829 supplemented with EDTA-free protease inhibitor cocktail (Roche). The nuclei were recovered
830 by centrifugation at 500 g for 5 min at 4°C and washed once in the same buffer. Nuclei were
831 resuspended in 100 µL FA-lysis buffer (50 mM HEPES/KOH (pH 7.5), 140 mM NaCl, 1 mM
832 EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitor cocktail) containing
833 1% SDS and incubated for 10 min at RT. After addition of another 100 µL FA-lysis buffer,
834 the mixture was transferred to 1.5 ml bioruptor microtubes (Diagenode) and sonicated using a
835 Bioruptor Pico water bath sonicator (Diagenode) for 10 cycles (30 s ON and 30 s OFF, 4°C).
836 The sonicated lysates were clarified by centrifugation at 16,000 g for 10 min. The
837 supernatants were collected and 20 µL (1/10) was set aside as input controls. The rest was
838 mixed in 800 µl FA-lysis buffer supplemented with protease inhibitors with 40 µL packed
839 bead volume of protein A-Sepharose CL-4B (GE Healthcare) coupled to 4 µL anti-HA
840 antibodies (HA.11, clone 16B12, ENZ-ABS118, Enzo Life Sciences) and pre-incubated in
841 FA-lysis buffer with sonicated salmon sperm DNA and BSA. After an overnight incubation at
842 4°C under constant rotation, the beads were sedimented by brief centrifugation. The beads
843 were washed twice with 1 mL FA-lysis buffer, twice with high-salt buffer (50 mM HEPES-
844 KOH (pH 7.5), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate),
845 twice with LiCl buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 1% NP-40,
846 and 1% sodium deoxycholate) and once with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM

847 EDTA). Bound protein–DNA complexes were eluted from beads by incubation for 10 min at
848 65°C in 200 µL elution buffer (50 mM Tris-HCl (pH 8.0), 1% SDS, 10 mM EDTA). After
849 addition of 200 µL TE buffer and 60 µg proteinase K (Eurobio Scientific), DNA crosslinks
850 were reversed by overnight incubation at 65°C. Samples were extracted twice with phenol–
851 chloroform, once with chloroform, and then ethanol precipitated and resuspended in TE
852 buffer. The input DNA treated identically and the recovered DNA were quantified by real-
853 time PCR using the KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (Kapa
854 Biosystems) and the Bio-Rad CFX96 Real-time PCR System. The values shown in the figures
855 are the ratios between the ChIP signals and the respective input DNA signals. All data are
856 representative of at least two independent experiments. The oligonucleotide primers used are
857 listed in Supplementary Table 3.

858 ***Co-Immunoprecipitation***

859 Whole cell extracts were prepared from the same HepG2 cells expressing HA-tagged proteins
860 in a CRISPR/Cas9 knockout background as used in the ChIP experiments shown in [Figs 1c](#)
861 and [2c](#). About $3\text{-}4 \times 10^6$ cells were collected from a 10 cm diameter dish using trypsin-EDTA
862 and pelleted by low-speed centrifugation. Cell were washed once with ice-cold PBS and
863 resuspended in 400 µL of co-immunoprecipitation (co-IP) lysis buffer (50 mM HEPES-KOH
864 (pH 7.5), 150 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 50 µM ZnCl₂, 0.1% IGEPAL (Sigma-
865 Aldrich CA-630)) supplemented with protease inhibitor cocktail (Roche). The lysates were
866 clarified by centrifugation at 16 000 g for 20 min at 4°C. The supernatants were collected and
867 40 µl (1/10) was set aside as input controls. The rest was mixed in 600 µl of co-IP lysis buffer
868 with 40 µL packed-bead volume of protein A-Sepharose CL-4B (GE Healthcare) coupled to 3
869 µL of anti-HA antibody (HA.11, clone 16B12, ENZ-ABS118, Enzo Life Sciences). After 4 h
870 of incubation at 4°C under constant rotation, the beads were sedimented by brief
871 centrifugation (1 min at 300 g) and the supernatant was discarded. The beads were washed 3

872 times with 1 mL of co-IP lysis buffer. Bound protein-protein complexes were released from
873 the beads by addition of 20 μ L of 2 \times Laemmli SDS sample buffer. The inputs and eluted
874 proteins were analysed by immunoblotting.

875 ***Colony Formation Assay***

876 HepG2 cells were transduced with puromycin-resistant plentiCRISPR v2 lentiviruses
877 expressing gene-specific sgRNAs or no sgRNA (Ctrl) as indicated in the figures. Four days
878 after transduction, cells were selected with 5 μ g/mL puromycin (Invivogen). One day later,
879 cells were seeded at low density in twelve-well plates, transduced with lentiviral constructs
880 encoding the indicated proteins, and further cultured for 14-21 days under puromycin
881 selection. Resistant colonies were fixed and stained with 0.05% crystal violet.

882 ***RNA interference and HBV infection of PHH***

883 PHH were transfected with 10-50 nM siRNA using Lipofectamine RNAiMax according to the
884 manufacturer's instructions (Life Technologies). The PHH media was refreshed 24 hours post-
885 transfection. For confocal microscopy, PHH were further incubated for 13 days before fixing
886 and staining. In [Extended Data Fig. 1a](#), PHH were incubated for 3 days post-transfection
887 before infecting with wild-type (WT) or HBx-deficient (Δ X) HBV (1,000 genomic
888 equivalents/cell) as described ⁴⁹. Production of HBV(WT) and HBV(Δ X) virions (genotype
889 D) from, respectively, HepAD38 and HepG2-H1.3x- stable cell lines was performed as
890 previously described ^{20,49}.

891 ***Immunostaining and Confocal Microscopy***

892 PHH were seeded onto glass coverslips (Corning BioCoat Poly-D-Lysine/Laminin 12 mm,
893 Corning, NY) in 12-well Corning CellBind plates. At the end of treatment, cells were fixed
894 with Perfusion Fixative Reagent (ThermoFisher Scientific) for 10 min and washed three times
895 in Dulbecco's Phosphate-Buffered Salt Solution (DPBS) (Corning). Immunostaining was

896 performed at RT. Cells were permeabilized in 0.3% Triton X-100 (MilliporeSigma) for 15
897 min following by blocking in DPBS with 3% bovine serum albumin (BSA) (MilliporeSigma)
898 and 10% HyClone Fetal Bovine Serum (FBS) (MilliporeSigma) for 60 min. The final
899 concentrations of primary antibodies were 0.2 µg/ml rabbit anti-Nse2 polyclonal antibody
900 (ThermoFisher Scientific, PA5-65676), 1 µg/ml mouse monoclonal anti-Smc6 antibody
901 (AT3956a), 4.44 µg/ml rabbit anti-PML antibody (Novus Biologicals, NB100-59787), 10
902 µg/ml mouse polyclonal anti-Sp100 antibody (Abcam, ab167605), and 1 µg/ml rabbit
903 polyclonal anti-SLF2 antibody (Abcam, ab122480). Secondary antibodies conjugated with
904 either Alexa Fluor 488 donkey anti-mouse IgG, ThermoFisher Scientific, A21202), or Alexa
905 Fluor 647 goat anti-rabbit IgG (H+L), ThermoFisher Scientific, A32733) were used at 2
906 µg/ml. All antibodies were diluted in DPBS supplemented with 1.5% BSA. Primary and
907 secondary antibodies were applied for 90 min and 60 min, respectively. The coverslips with
908 stained cells were mounted on glass microscopic slides (VWR International, Radnor, PA) by
909 addition of a drop of ProLong Diamond antifade reagent containing DAPI (ThermoFisher
910 Scientific, P36962). Samples were imaged with confocal laser scanning microscope Leica
911 SP8 (Leica Microsystems Inc., Wetzlar, Germany). All images within each sample set were
912 captured using identical instrument settings. Acquisition was performed in three sequences to
913 minimize bleed-through artifacts. DAPI was excited at 405 nm with UV laser and detected at
914 415-480 nm during the first sequence. Alexa Fluor 488 was excited at 499 nm and detected at
915 498-600 nm during the second sequence. Alexa Fluor 647 was excited at 647 nm and detected
916 at 667-800 nm. Image analysis was performed using Imaris 9.5.1 (Bitplane, Belfast, UK) with
917 nuclear detection based on DAPI staining.

918 ***ELISA***

919 Hepatitis B e antigen (HBeAg) was detected in culture media at the indicated time by
920 electrochemiluminescence assay (MSD). The MSD assay was performed according to the

921 manufacturer's instructions (Meso Scale Diagnostics, Rockville, MD). Briefly, cultured
922 supernatants were inactivated with 0.5% Triton X-100 (30 minutes at 37°C) and then
923 transferred into plates pre-spotted with an anti-HBeAg antibody (Genway Bio, San Diego,
924 CA). The plates were then incubated for 2 hours at room temperature with gentle shaking,
925 followed by a wash step in PBS with 0.5% Tween. MSD Sulfate tags anti-A and anti-B (1
926 µg/ml each) were then added to the wells and the plates incubated for a further 2 hours at
927 room temperature with gentle shaking, followed by another wash step in PBS with 0.5%
928 Tween. A 2X solution of MSD T Buffer Read was then added and the plate was read on a
929 Sector Imager 6000 plate scanner.

930 ***RT-PCR Analysis***

931 Total cellular RNA was isolated from PHH cultured in 96-well plates using an RNeasy 96 Kit
932 (Qiagen) following the manufacturer's instructions. Real-Time RT-PCR was performed with
933 TaqMan1 Fast Virus 1-Step Master Mix (Life Technologies) using a QuantStudio 7 Flex
934 Real-Time PCR System (Life Technologies) following the manufacturer's instructions. β-actin
935 mRNA expression was used to normalize target gene expression. All oligonucleotide primer
936 sets were manufactured by Life Technologies.

937 ***Statistical analysis***

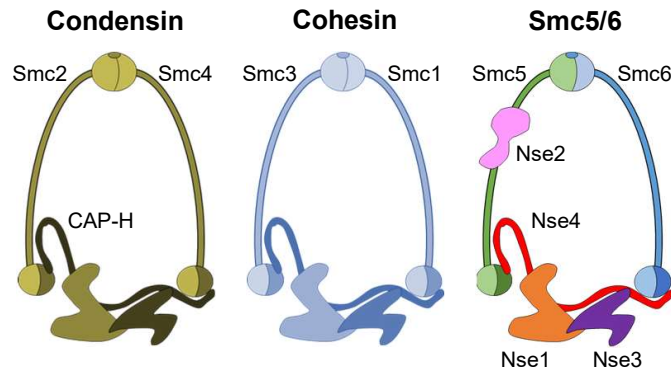
938 Statistical significance of log-transformed data was tested using a two-tailed, paired t-test (for
939 two sample comparisons) or unpaired one-way ANOVA with Dunnett's multiple comparison
940 correction (for multiple comparisons). A value of $p < 0.05$ was considered significant.

941

942 **Data availability**

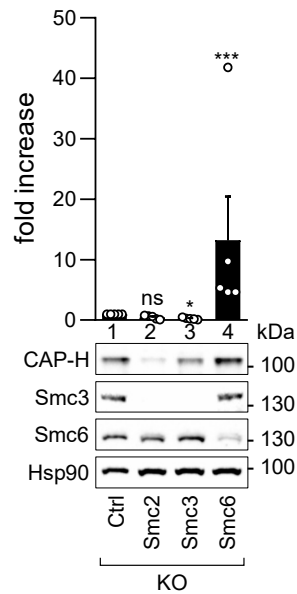
943 All data are available from corresponding authors upon reasonable request.

A

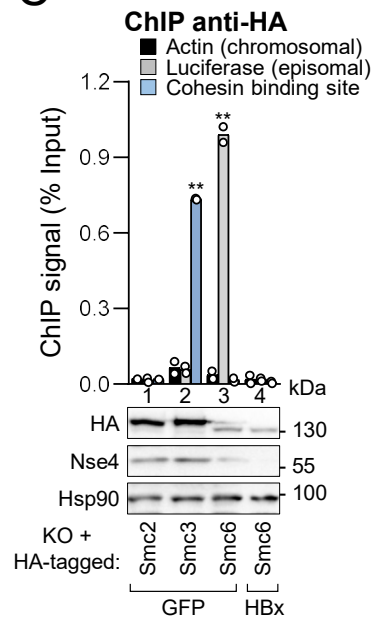


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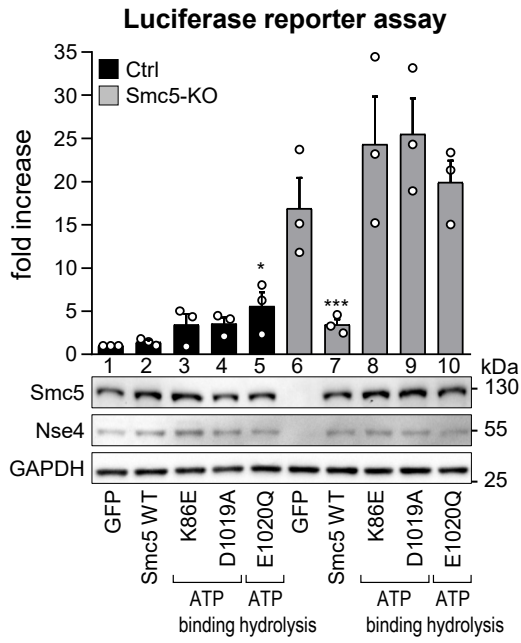
Luciferase reporter assay



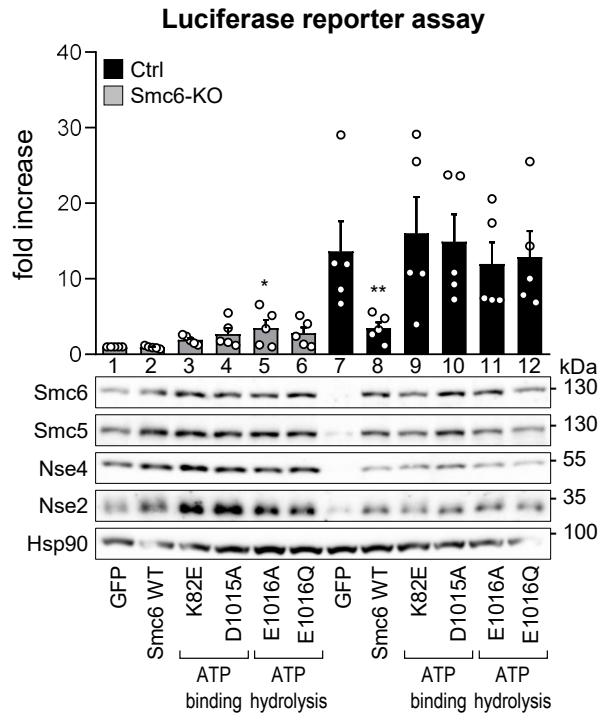
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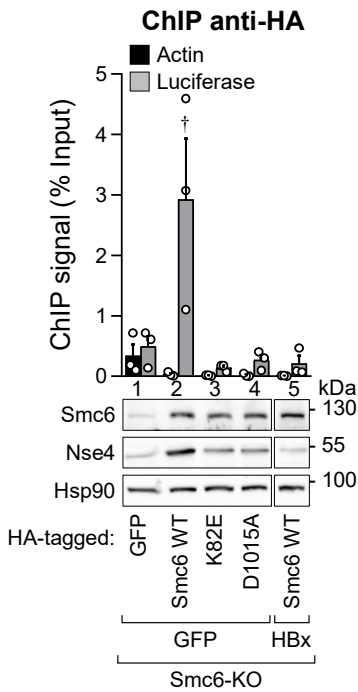
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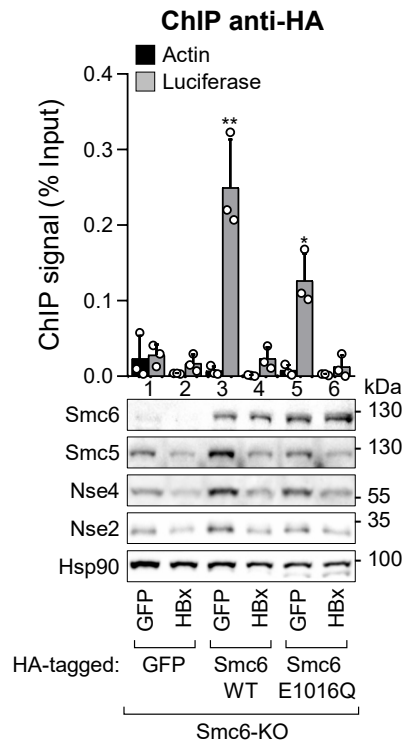
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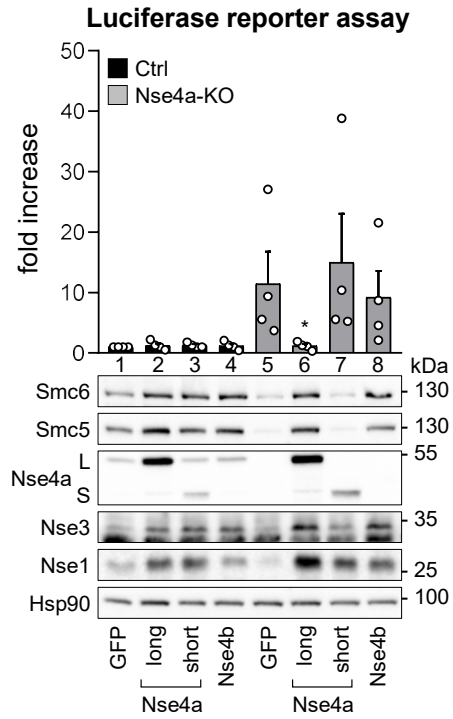
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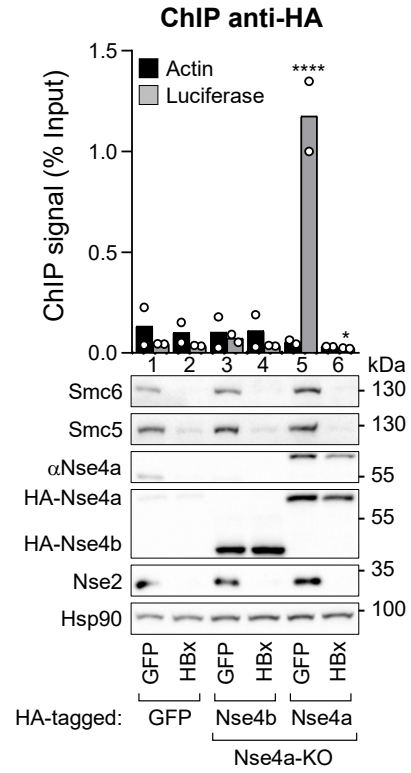
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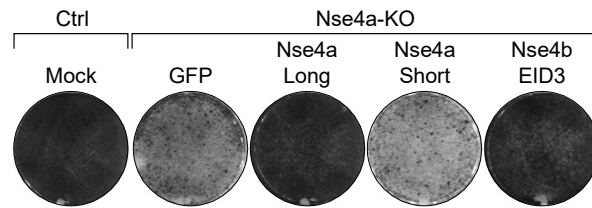
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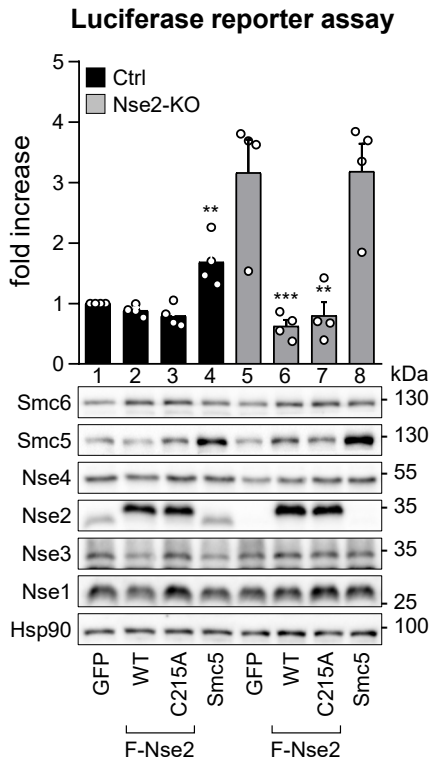
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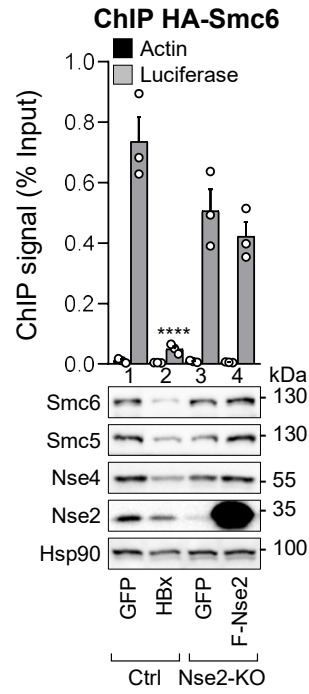
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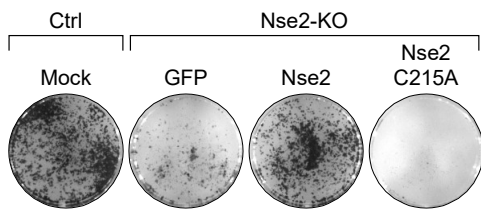
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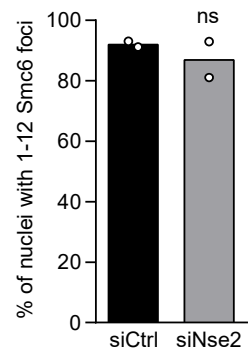
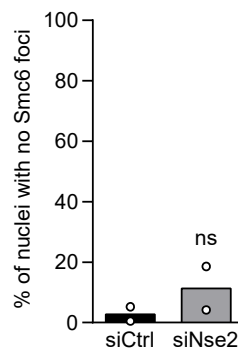
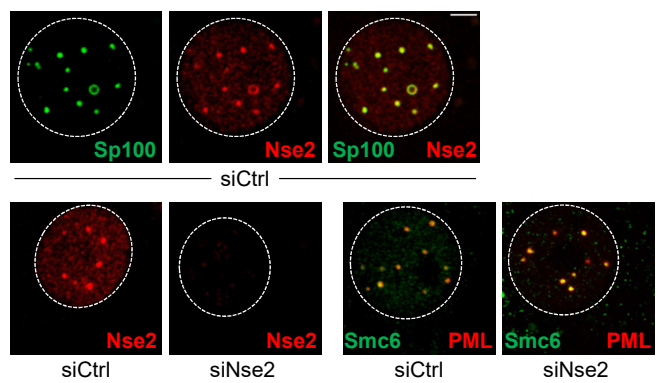
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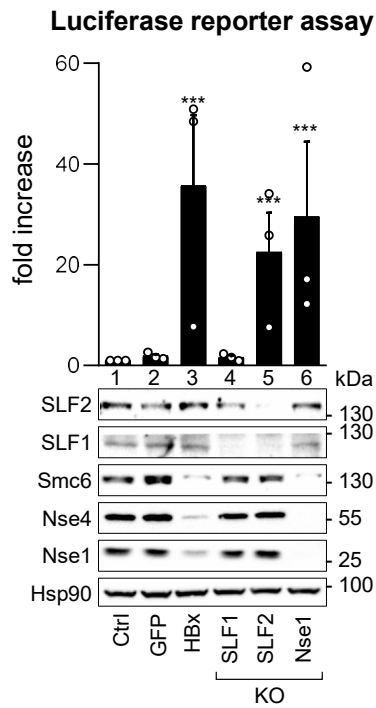
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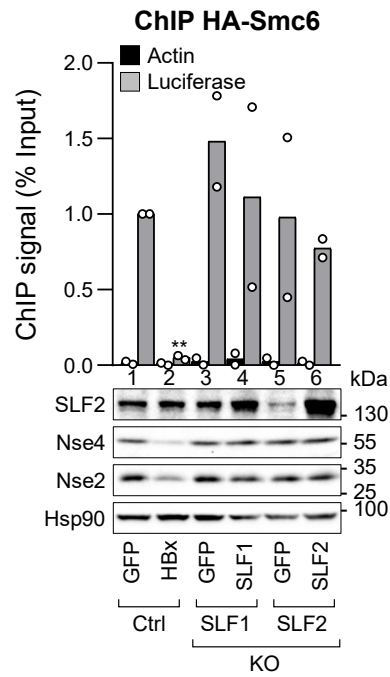
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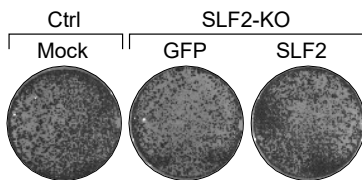
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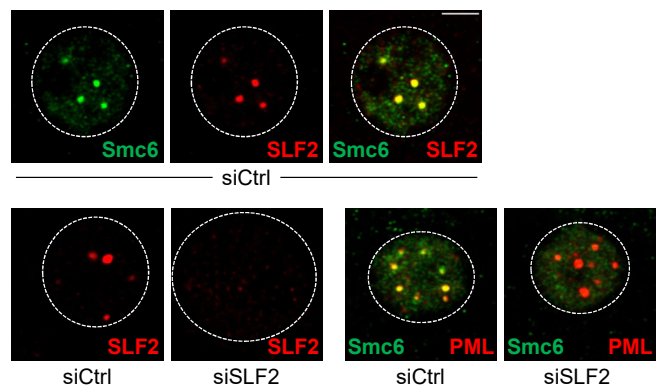
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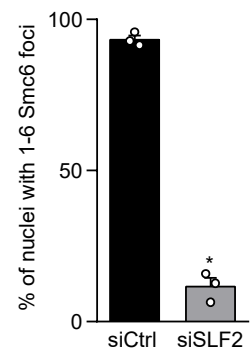
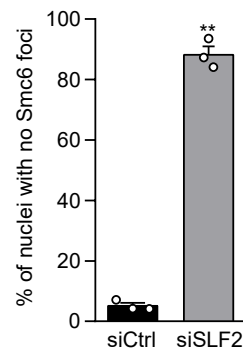
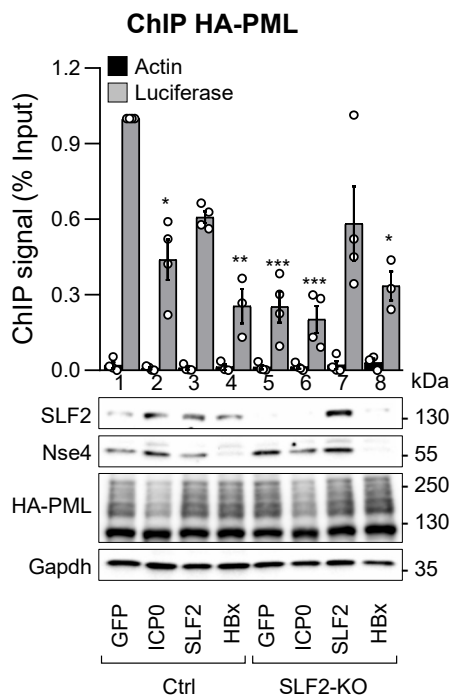
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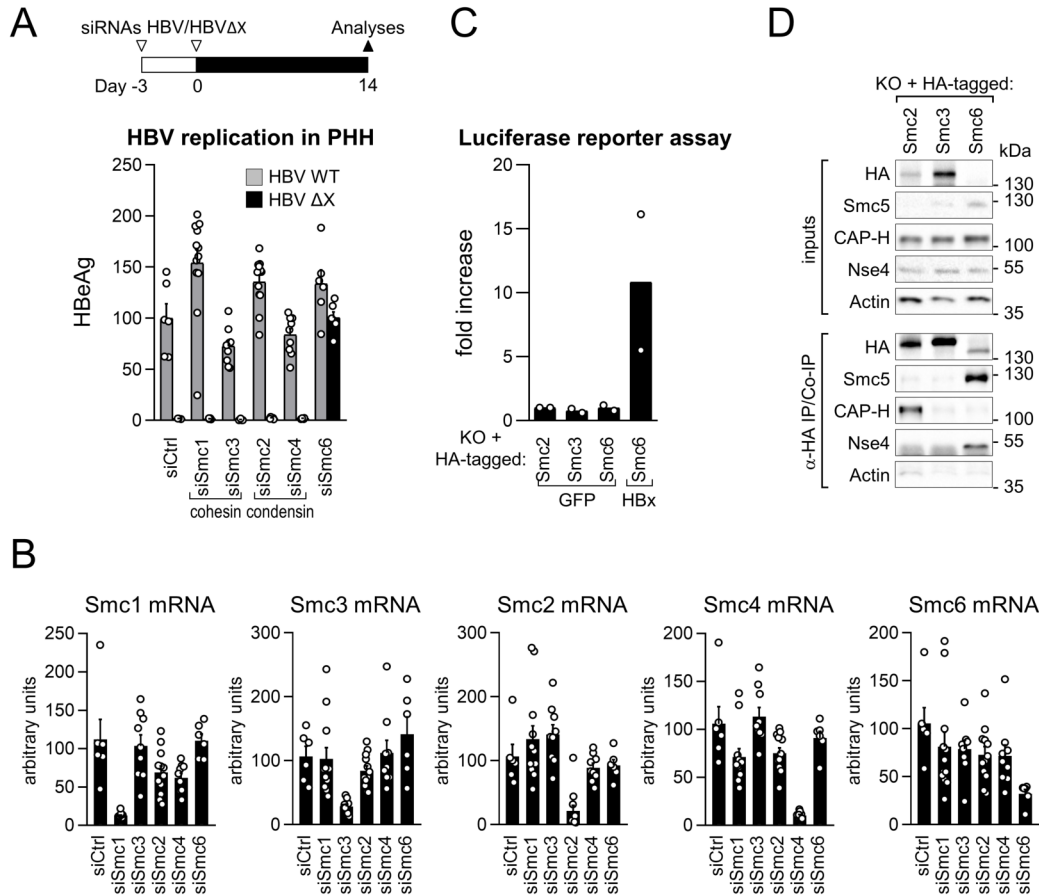


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E





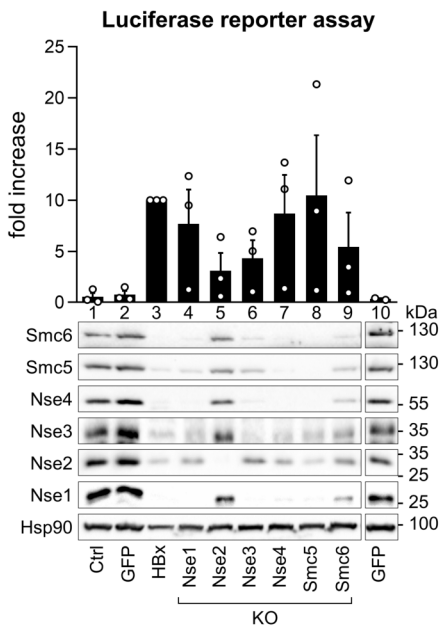
Extended Data Figure 1. Smc5/6 is the only SMC complex to silence expression of the episomal HBV genome. Related to Figure 1.

a, PHH were transfected with a non-targeting control siRNA (siCtrl) or with siRNA targeting the indicated Smc subunits. Cells were then incubated for 3 days and infected with wild-type (WT) or an HBx-deficient (Δ X) HBV. HBeAg secretion, a marker for HBV gene expression, was measured 14 days later. HBeAg levels are expressed as a percentage of those in control cells infected with HBV(WT) (grey bars) or in siSmc6-treated cells infected with HBV(Δ X) (black bars). Data are means \pm SEM of three independent experiments with samples from one PHH donor. The schematic diagram summarizing the design of the study is shown at the top.

b, The mRNA levels of the indicated Smc proteins in the samples analyzed in **a** were determined by real-time RT-PCR and normalized to β -actin. Values are expressed as a percentage of those in control cells (siCtrl). Data are means \pm SEM of three independent experiments with one PHH donor.

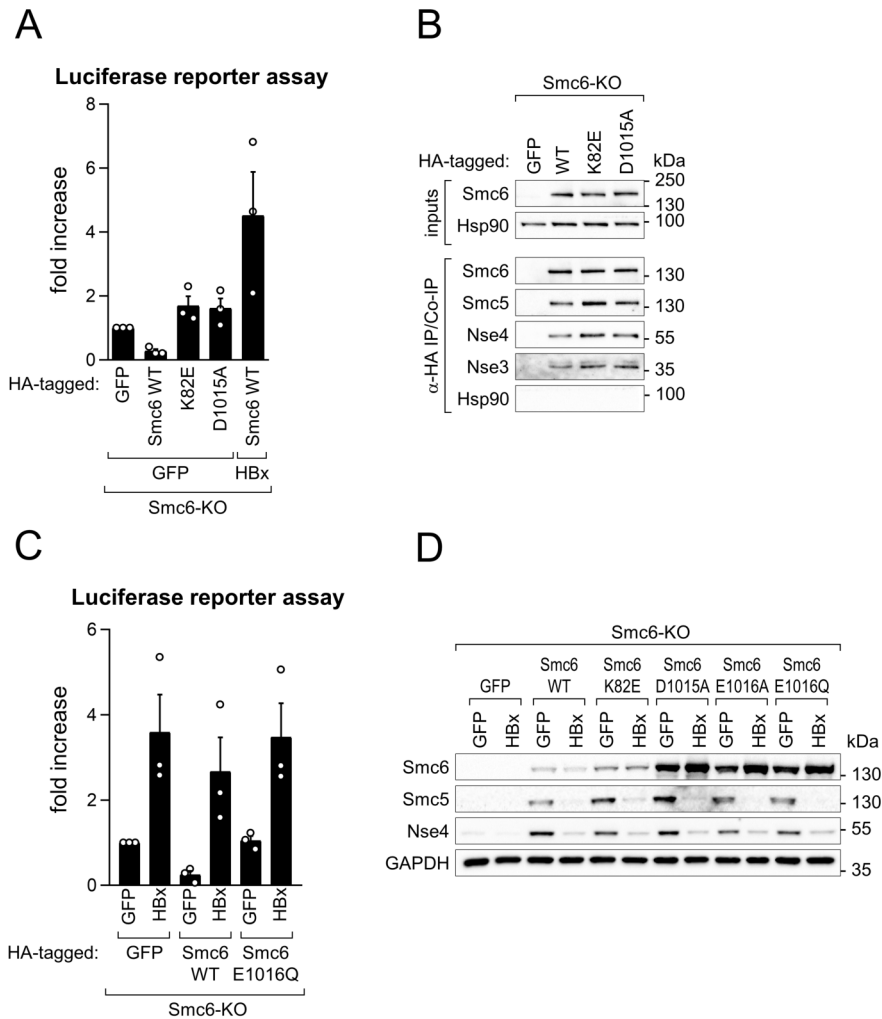
c, Luciferase assay for the ChIP experiment presented in Fig. 1c. Data are means \pm SEM of 2 independent experiments.

d, HA-Smc2 assembles into a condensin complex. Whole extracts prepared from HepG2 cells expressing the indicated HA-tagged SMC protein in a CRISPR/Cas9 knockout background were immunoprecipitated with anti-HA antibodies. The amounts recovered and the presence of Smc5, CAP-H and Nse4 in the eluates were assessed by Western blotting. CAP-H and Nse4 are the kleisin subunits of, respectively, condensin (Smc2) and the Smc5/6 complex. See Fig. 1a. Actin serves as a negative control. The experiment was repeated twice independently with similar results.



Extended Data Figure 2. Effect of single-subunit depletion on Smc5/6 complex integrity and restriction activity.

HepG2 cells were transduced with lentiviral constructs expressing Cas9 alone (Ctrl) or Cas9 together with sgRNA targeting the indicated Smc5/6 subunits, or with lentiviruses encoding GFP or GFP-HBx, as indicated. After selection, cells were transfected with a luciferase reporter plasmid. Luciferase assay and Western blot analysis were performed 5 days later. Hsp90 serves as a loading control. Unessential lanes were removed from the original blot images. Luciferase activity is relative to that measured in cells expressing HBx, which was set to 10. Data are means \pm SEM of 3 independent experiments.



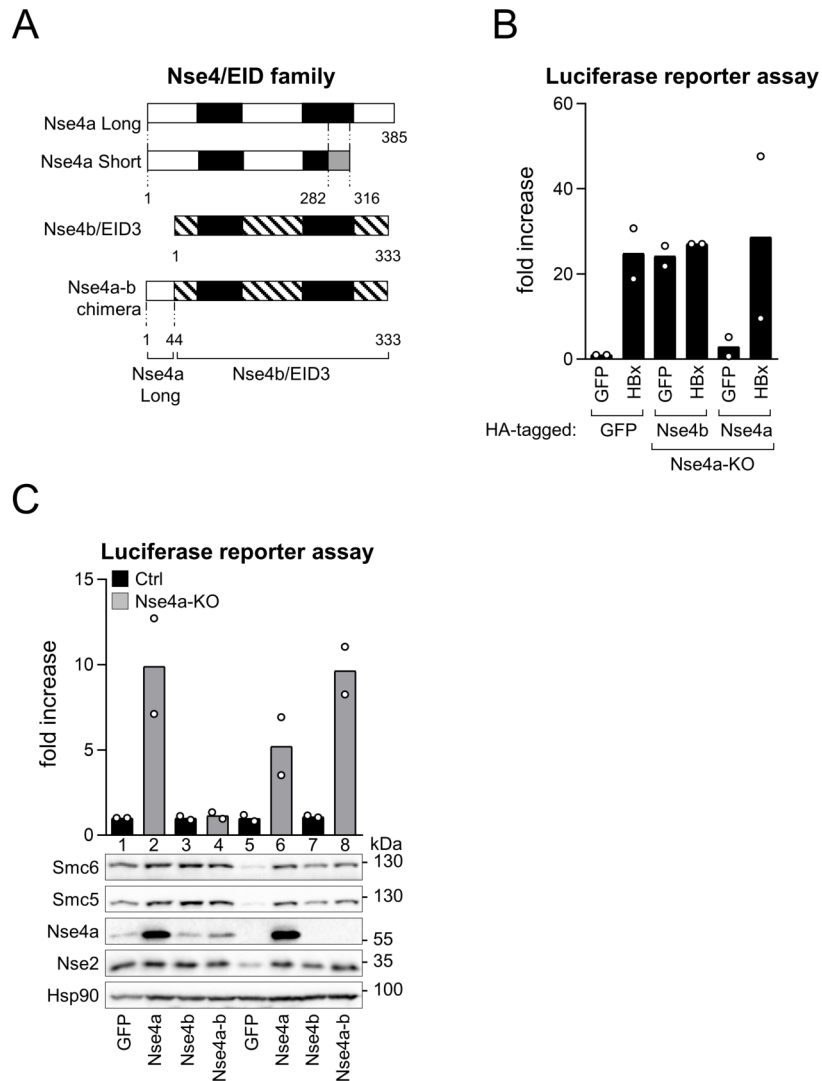
Extended Data Figure 3. Involvement of Smc5 and Smc6 ATP binding and hydrolysis in Smc5/6 episomal DNA binding and restriction, and degradation by HBx. Related to Figure 2.

a, Luciferase assay for the ChIP experiment presented in Fig. 2c. Data are means \pm SEM of 3 independent experiments.

b, Smc6 ATP binding mutants normally assemble into Smc5/6 complexes. Whole extracts prepared from HepG2 cells depleted of Smc6 and expressing HA-GFP or the indicated HA-tagged SMC protein were immunoprecipitated with anti-HA antibodies. The amounts recovered and the presence of other Smc5/6 subunits in the eluates were assessed by Western blotting. Hsp90 serves as a negative control. The experiment was repeated twice independently with similar results.

c, Luciferase assay for the ChIP experiment presented in Fig. 2d. Data are means \pm SEM of 3 independent experiments.

d, HepG2 cells depleted of Smc6 and expressing GFP or the indicated wild-type or mutant Smc6 proteins from lentiviral vectors exactly as in Fig. 2b were also transduced with GFP or GFP-HBx. Western blot analysis was performed as before. GAPDH serves as a loading control. The experiment was repeated thrice independently with similar results.

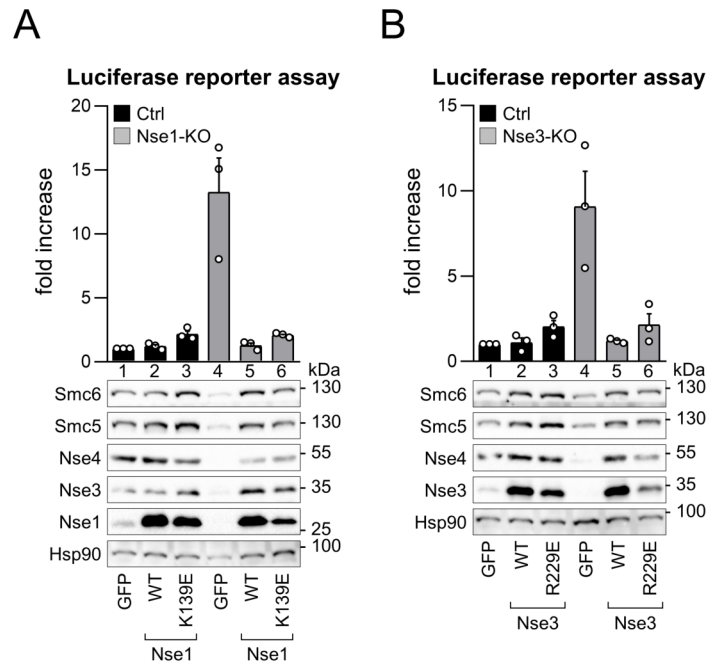


Extended Data Figure 4. Nse4a performs an essential function in Smc5/6 restriction for which Nse4b cannot substitute. Related to Figure 3.

a, Schematic diagram of Nse4a (long), the shorter isoform of Nse4a, Nse4b and the Nse4b variant bearing the N-terminal region unique to Nse4a. White boxes indicate Nse4a sequences and the region of the short Nse4a protein common to both splicing isoforms. Hatched boxes indicate regions of Nse4b showing homology to Nse4a. The grey box indicates a region with no homology. Highlighted in black are the highly conserved N-terminal and C-terminal kleisin domains that have the potential to form helix-turn-helix and winged-helix motifs and are involved in Nse4 interaction with Smc6 and Smc5, respectively (Palecek et al., 2006; Vondrova et al., 2020).

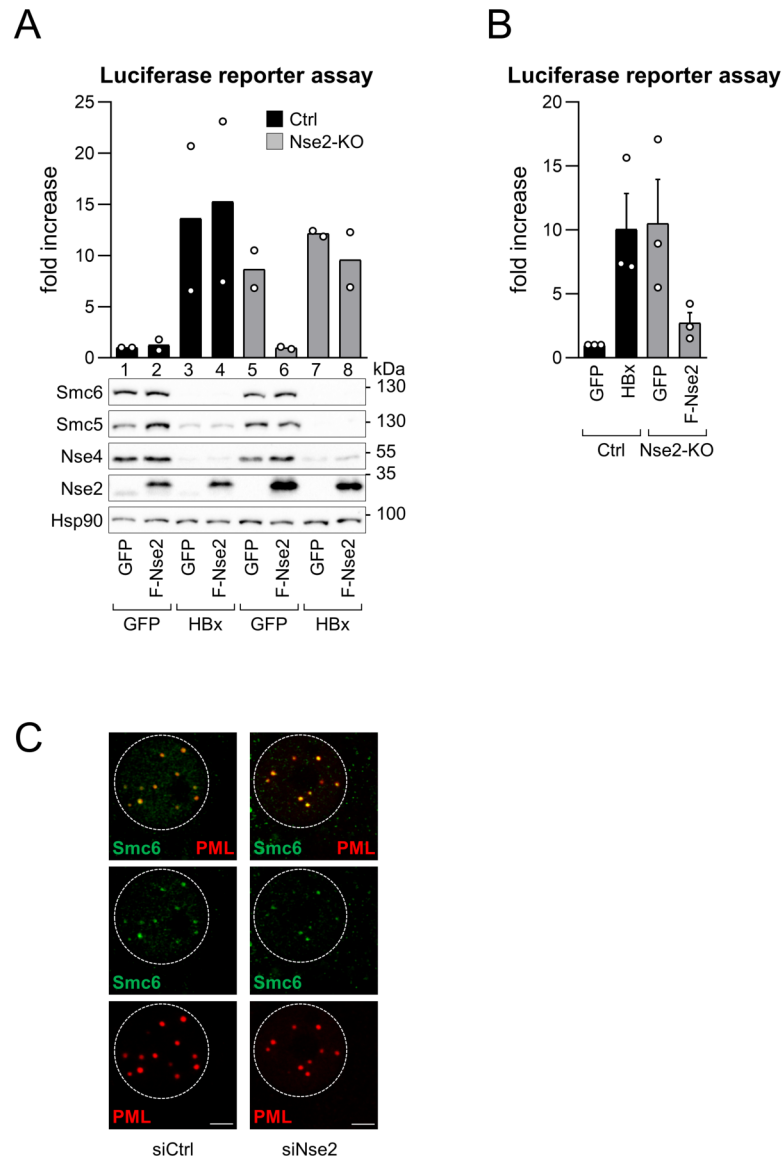
b, Luciferase assay for the ChIP experiment presented in Fig. 3b. Data are means \pm SEM of 2 independent experiments.

c, Same experiment as in Fig. 3a but including an Nse4b chimeric protein carrying the N-terminal region unique to Nse4a (Nse4a-b; see panel **a**). Expression of Nse4b and Nse4a-b was inferred from their stabilization effect on the other Smc5/6 subunits (lane 8). Data are means \pm SEM of 2 independent experiments.



Extended Data Figure 5. Nse1 and Nse3 DNA-binding mutants are functional in vivo.

a,b, Control HepG2 cells (black bars) and cells depleted of Nse1 (**a**) or Nse3 (**b**; grey bars) were transfected with a luciferase reporter plasmid and shortly after transduced with GFP or the corresponding wild-type or DNA-binding mutant protein as indicated (Zabradý et al., 2016). Luciferase assay and Western blot analysis were as before. Data are means \pm SEM of 3 independent experiments.

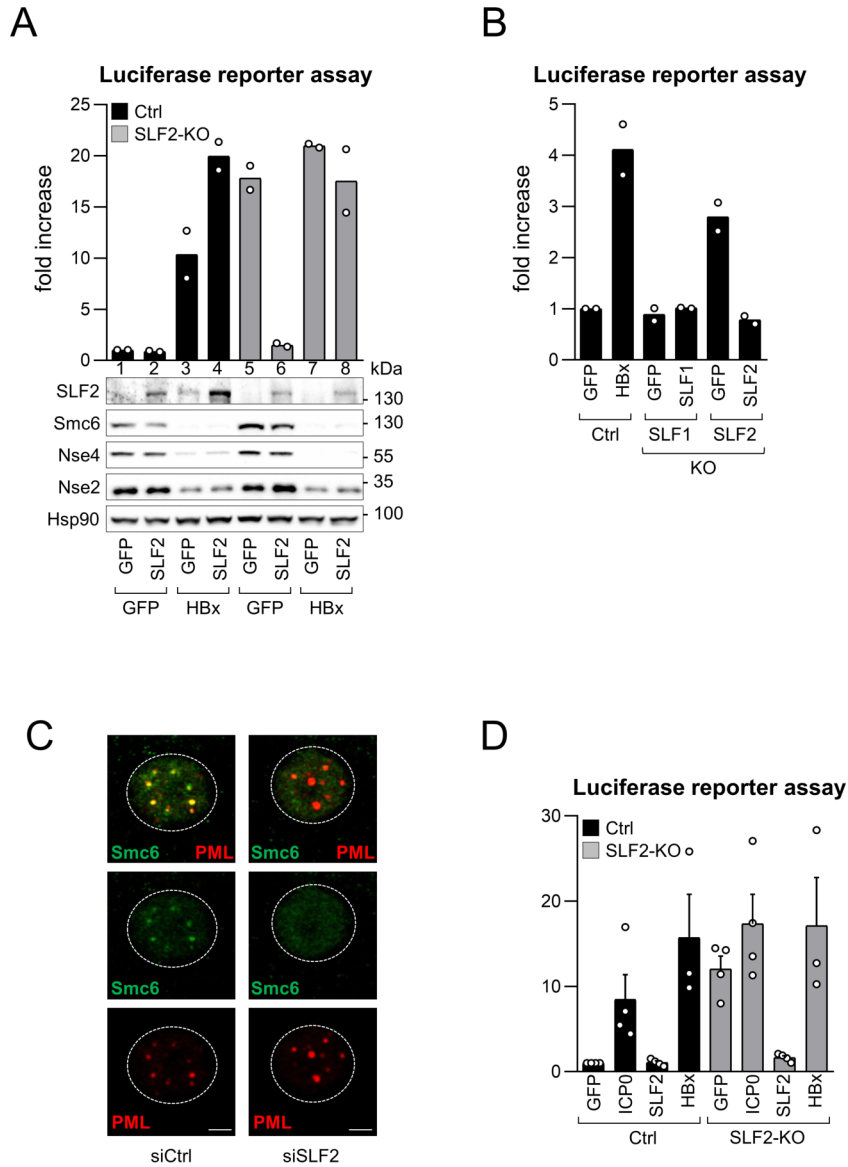


Extended Data Figure 6. HBx triggers Smc5/6 degradation in the absence of Nse2. Related to Figure 4.

a, Control HepG2 cells (black bars) and cells depleted of Nse2 (grey bars) were transfected with a reporter gene and transduced with GFP or Flag-tagged Nse2 (F-Nse2). Cells were then split and further transduced with GFP or GFP-HBx. Luciferase assay and Western blot analysis were as before. Data are expressed as mean \pm SEM of 2 independent experiments.

b, Luciferase assay for the ChIP experiment presented in Fig. 4b. Data are expressed as mean \pm SEM of 3 independent experiments.

c, Single channel confocal images of middle right panels merged images presented in Fig. 4d.



Extended Data Figure 7. HBx triggers Smc5/6 degradation in the absence of SLF2. Related to Figure 5.

a, Control HepG2 cells (black bars) and cells depleted of SLF2 (grey bars) were transfected with a reporter gene and shortly after transduced with GFP, GFP-HBx and/or SLF2 in the indicated combinations. Luciferase assay and Western blot analysis were performed as before. Data are expressed as mean \pm SEM of 2 independent experiments.

b, Luciferase assay for the ChIP experiment presented in Fig. 5b. Data are means \pm SEM of 2 independent experiments.

c, Single channel confocal images of middle right panels merged images presented in Fig. 5d.

d, Luciferase assay for the ChIP experiment presented in Fig. 5e. Data are means \pm SEM of 4 independent experiments.