1 Cohesin mutation sensitizes cancer cells to anti-PD-1 therapy through 2 endogenous retrovirus-mediated PD-L1 upregulation

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31

32 **Abstract**

33 Immune checkpoint therapy shows impressive and durable clinical responses in
34 cancer patients, but the genetic determinants that enable cancer cells to respond to
35 anti-PD-1 therapy are still elusive. Herein, we identified that *NIPBL* deficiency
36 promotes endogenous retrovirus (ERV) expression in tumour cells, which in turn
37 inactivates CD8⁺ tumour-infiltrating lymphocytes (TILs) via the PD-L1/PD-1
38 inhibitory checkpoint pathway. Mechanistically, *NIPBL* deficiency impairs DNMT1
39 transcription, preventing DNMT1 from suppressing ERV expression in tumour cells;
40 ERVs stimulate PD-L1 expression by inducing the STAT2-IRF9 complex, a
41 downstream event of double-stranded RNA (dsRNA)-MAVS-IRF3 signalling, and
42 thereby suppress CD8 TIL-mediated immunity. An anti-PD-1 monoclonal antibody
43 achieved remarkable therapeutic effects in *Nipbl*-deficient syngeneic tumour models
44 and improved host survival by eliciting an antitumour memory immune response.
45 Cancer patients harbouring mutations of cohesin subunits and regulators plus DNMT1
46 had significantly better responses to anti-PD-1 therapy than their non-mutated
47 counterparts did. Our study reveals a novel mechanism by which cohesin complex
48 deregulation stimulates ERV expression by impairing DNMT1 expression and fosters
49 an immunosuppressive tumour microenvironment by activating the PD-L1/PD-1
50 inhibitory checkpoint.

51

52 Main

53 Immune inhibitory checkpoint molecules play a critical role in tumour
54 development. For example, programmed cell death ligand-1 (PD-L1) is a ligand for
55 the PD-1 receptor, which is highly expressed in tumours. PD-L1 blocks the activation
56 of T cell receptors when it binds to the PD-1 receptor and eventually enables tumour
57 cells to escape from the antitumour immune response mediated by cytotoxic CD8+ T
58 cells¹⁻³. Anti-PD-1 monoclonal antibodies (mAbs), such as nivolumab and
59 pembrolizumab, have achieved remarkable, persistent clinical outcomes in patients
60 with melanoma, non-small-cell lung cancer and tumours with microsatellite instability
61 (MSI)⁴⁻⁷. However, only a limited subpopulation of patients benefit from immune
62 checkpoint block therapies^{8,9}. The genomic determinants of the response to anti-PD-1
63 therapy remain unknown.

64 To explore the genetic determinants for responding to anti-PD-1 therapy, we
65 performed an *in vivo* genome-scale CRISPR-Cas9 knockout (GeCKO) screening,
66 which revealed that loss of cohesin subunits and regulators increased tumour
67 sensitivity to anti-PD-1 therapy by stimulating PD-L1 expression in tumour cells.
68 Cohesin is a multiple subunit complex composed of core subunits (SMC1A/SMC1B,
69 SMC3, STAG1/STAG2 and RAD21) and regulators (NIPBL, PDS5A/PDS5B, WAPL,
70 CDCA5 and MAU2)¹⁰. NIPBL is a key factor for loading the cohesin complex at
71 promoters. Apart from its role in sister chromatid cohesion, the cohesin complex has
72 been implicated in 3D genome organization and gene regulation¹¹⁻¹⁵. Recently,
73 pan-cancer atlas analysis identified the cohesin complex as one of 16 most commonly

74 mutated subnetworks in human cancer^{15, 16}. However, it is unclear whether the cohesin
75 complex functions by regulating immunity in cancer, and the immune-regulatory role
76 of the cohesin complex remains largely unknown.

77 In this study, we reveal that knockout of *Nipbl* and other cohesin complex members
78 stimulates dsRNA ERV expression by blocking DNMT1 transcription, which in turn
79 boosts PD-L1 expression by activating the dsRNA-MAVS-IRF3-STAT2/IRF9
80 signalling pathway in tumour cells. Blockade of PD-1 using a specific monoclonal
81 antibody resulted in 70% of *Nipbl*-deficient tumours completely regressing,
82 remarkably improving mouse survival by reactivating cytotoxic CD8+ T cells.
83 Mutations in cohesin subunits and regulators plus DNMT1 can predict the survival
84 rate of melanoma, lung and colorectal cancer patients compared with that of their
85 non-mutated counterparts. In summary, our study reveals a novel mechanism by
86 which loss of cohesin subunits and regulators in tumours shapes the tumour-immune
87 cell interaction and indicates that tumours with these mutations could be selectively
88 treated with anti-PD1 therapy.

89

90 **Results**

91 ***In vivo* genome-scale screening reveals that *Nipbl*-deficient tumours potentially**
92 **respond to anti-PD-1 therapy.**

93 To identify genetic determinants in the response to anti-PD-1 therapy, we selected
94 an immunotherapy-insensitive murine CT26 colorectal cancer cell line to perform
95 GeCKO screening *in vivo*¹⁷⁻¹⁹. CT26 cells were infected with lentivirus containing a

96 library of single guide RNAs (sgRNAs) at a multiplicity of infection (MOI) of 0.3 and
 97 then selected the cells for 7 days with puromycin to remove uninfected cells. The
 98 transduced cells were subcutaneously injected into the flanks of syngeneic mice
 99 (2×10^7 cells per injection, 10 mice per group), which were then intraperitoneally
 100 administered either isotype IgG or anti-PD-1 monoclonal antibody five times (200
 101 $\mu\text{g}/\text{mouse}$ per injection, Fig. 1A). After treatment stopped, genomic DNA was
 102 extracted from each tumour sample, normalized and subjected to barcoded deep
 103 sequencing (see more information in the Material and Methods).

104 T cell-based CRISPR-Cas9 screens have been described by several laboratories.
 105 For example, mutations of the phosphatase *Ptpn2* and the G protein-coupled receptor
 106 *APLNR* confers tumour cells resistant to T-cell-mediated cytotoxicity^{20, 21}; mutations
 107 of SWI/SNF complex genes sensitize tumour cells to immunotherapy²². Recently, *Fli1*
 108 is reported as a key negative regulator of cytotoxic CD8⁺ T cells²³. Our approach
 109 emphasized sensitive selection of sgRNAs depleted by an *in vivo* anti-PD-1 therapy,
 110 which allowed us to specifically identify cancer mutations conferring sensitivity to
 111 anti-PD-1 therapy.

112 After normalized by medium normalization, the significant distribution of raw read
 113 counts in different groups were calculated by the MAGeCK algorithm (false
 114 discovery rate < 0.05)²⁴, and the fold changes were calculated by comparing read
 115 counts of anti-PD-1 group to that of control IgG group (Fig. 1B). We used the
 116 following criteria to select sensitive candidates: 1) was one of the top 6,000 reduced
 117 sgRNAs by comparing the anti-PD-1 group to the control group; 2) was one of sixteen

commonly mutated subnetworks in human cancer¹⁶; and 3) had a minimum of >3 members per complex hit in a single screen with p value < 0.005 and fold change <0.03. As a positive control, sgRNAs targeting the SWI/SNF complex (*Pbrm1*, *Brd7*, *Arid2*, *Arid1a* and *Arid1b*), which were previously reported to mediate the immune response to anti-PD-1 therapy, were significantly reduced in the anti-PD-1 group compared to the vehicle group (Figs. 1B and S1A)²². As previously reported^{20, 22}, glycolysis genes such as *Gale* and *Nsdhl* as well as the phosphatase *Ptpn2* were also obviously depleted in our screening (Fig. S1B and S1C). Strikingly, sgRNAs targeting cohesin subunits and regulators were significantly reduced in the anti-PD-1 group compared to that of the control IgG group (Fig. 1B). For example, sgRNAs targeting cohesin complex subunits (*Stag1*, *Smc1b* and *Rad21l*) and regulators (*Pds5a* and *Escal*) were significantly reduced in the anti-PD-1/vehicle group compared to nontargeting sgRNA group (p<0.005, Fig. S1D). *Smc1b* and *Rad21l* are meiotic-specific cohesin subunits, which are actively expressed in many tumour types^{25, 26}.

As one of most commonly mutated cancer subnetworks, cohesin complex subunits and regulators were widely mutated in approximately 29.8% of 10,967 pan-cancer cases of thirty-two human cancer types and well correlated with patients' overall, progression-free and disease-free survivals (Figs. 1C, 1D and S2)^{15, 16, 27-29}. Notably, *NIPBL*, a key factor for loading the cohesin complex¹¹, ranked as the top candidate with an approximately 7% mutation rate in all human cancer types. For *NIPBL*, there were 124 cases of amplification (copy number ≥ 4 , 16.7%), 159 cases of truncation

(21.4%), 424 cases of missense (57.1%) and other mutations. Remarkably, cohesin complex subunits and regulators were inactively mutated in tumour samples that were PD-L1-positive ($\log_2(\text{RPKM}) > 0.5$) across eighteen cancer types from The Cancer Genome Atlas (TCGA), especially in stomach and colorectal tumours (Fig. 1E)^{27, 30}. Patient survival analysis revealed that anti-PD-1 therapy significantly prolonged the survival rate of lung cancer patients with mutations of cohesin subunits and regulators compared to that of patients without mutations ($p=0.012$, Fig. 1F and Supplemental Table 1)³¹. These data indicate that deficiency of cohesin subunits and regulators such as *NIPBL* potentially sensitizes tumour cells to anti-PD-1 therapy.

***Nipbl* loss facilitates tumour progression by inactivating CD8+ T cells through PD-1/PD-L1 inhibitory checkpoint.**

The inactivating mutation of *NIPBL* is well-documented with poorly characterization of immunomodulatory function³²⁻³⁴, so we chose it for further studies. Flow cytometry data illustrated that cell surface PD-L1 expression was significantly increased in CT26 (ratio=2.69, $p=4.5\text{E-}5$) and colon26 (ratio=4.02, $p=1.1\text{E-}3$) murine syngeneic colorectal tumour models when *Nipbl* was knocked out (Fig. 2A). Non-targeting sgRNA (sgControl) was used as a negative control. The PD-L1 mRNA and protein expression levels of sgNipbl CT26 and colon26 murine colorectal tumour cells were robustly increased compared to those of sgControl cells (Fig. 2B and 2C). Similar results were obtained using the HCT-116 human colorectal cancer cell line (Fig. 2D). The sgRNA-mediated knockout of *Nipbl* in CT26, colon26 and HCT-116 cells was validated by Sanger sequencing (Fig. S3A-S3C). Notably, high

162 PD-L1-expressing B16F10 and cloudman S91 murine tumour cell lines had little or
163 no detectable Nipbl protein expression compared to that of *Nipbl* wild-type CT26
164 cells (Fig. 2E) ¹⁸. *NIPBL*^{p.K603fs}-mutant RKO human colorectal tumour cells also had
165 more potent PD-L1 expression than *NIPBL* wild-type HCT-116 cells (Fig. 2F and see
166 more cell lines in Fig. 5B).

167 PD-L1 expression in tumour cells often lead to the exhaustion of CD8 TILs by
168 activating the PD-1/PD-L1 inhibitory checkpoint³⁵. Tumour-infiltrating immune cell
169 data analysis³⁶ revealed that *NIPBL* mRNA expression levels in tumour cells were
170 negatively correlated with PD-1 mRNA levels in the infiltrating immune cells of
171 TCGA colon adenocarcinomas (n=457, p=3.33E-3, Fig. 2G). Immune cell subtype
172 analysis further revealed that the proportion of infiltrated CD8+ T cells was
173 significantly increased in *NIPBL*-mutant colon tumour samples compared to *NIPBL*
174 wild-type samples (p<0.01, Fig. 2H). These data suggest that *NIPBL* loss potentially
175 inactivates CD8+ T cells by promoting the PD-1/PD-L1 interaction.

176 To verify this hypothesis, we inoculated *Nipbl* knockout murine tumour cells into
177 the flanks of syngeneic mice and observed that the proportions of CD45+ and CD8
178 TILs were significantly increased in both sgNipbl CT26 and colon26 tumour tissues
179 compared to sgControl tumour tissues (p<0.05, Fig. 2I and 2J); importantly, the
180 portion of CD8 TILs but no other immune cell subtypes was significantly increased in
181 sgNipbl tumour tissues compared to sgControl tumour tissues (p<0.01, Fig. 2K).
182 Moreover, CD8 TILs expressing PD-1 but not Eomes, two markers of exhausted T
183 cells³⁷, were increased by two-to-threefold when *Nipbl* was knocked out (Figs. 2L,

184 2M and S4A), supporting the notion that *Nipbl* mutation blocks the antitumour
185 immunity of CD8 TILs via the PD-1/PD-L1 inhibitory checkpoint pathway.

186 Noteworthy, we observed weakly or no expression of Cas9 and the phosphorylation
187 of histone H2AX at Ser13 (γ H2AX)³⁸ in sgNipbl and sgControl CT26, colon26 and
188 HCT-116 tumour cells (Fig. S5A and S5B). Exogenously overexpressed Cas9 and
189 mitomycin C-induced γ H2AX were used as positive controls. There were no
190 significant change of the cytosolic dsDNA content of sgNipbl CT26 and colon26
191 compared to that of sgControl cells ($p>0.05$, Fig. S5C). Moreover, shSTING had no
192 effect on PD-L1 expression compared to scramble (Fig. S5D). These data suggest that
193 double-strand DNA breaks is not responsible for *Nipbl* loss-induced PD-L1
194 expression.

195 Next, we asked whether *Nipbl* loss promotes tumour growth in mice. Although
196 cohesin knockout mice are early embryonic lethal^{39, 40}, tissue-specific *Rad21*
197 knockout mice has a normal lifespan⁴¹. Thus, we established *Villin-Cre*⁺; *Nipbl*^{flox/flox}
198 mice to achieve *Nipbl* deletion in gastrointestinal epithelial cells in which *NIPBL* is
199 highly mutated^{32, 42}. We observed no tumour formation in mice when heterozygous or
200 homozygous *Nipbl* deletion was achieved in gastrointestinal epithelial cells; these
201 mice showed a normal lifespan and pregnancy similar to that in control *Nipbl*^{flox/flox}
202 mice ($p>0.05$, Fig. 2N). When crossed with *Apc*^{Min} mice, *Nipbl* loss significantly
203 promoted tumour progression by reducing the survival rate of these mice compared to
204 that of *Apc*^{Min} mice, and this change was accompanied by increased PD-L1 expression
205 ($p<0.0001$, Fig. 2O).

Collectively, we conclude that *NIPBL* loss promotes tumour progression by promoting the PD-L1/PD-1 interaction.

***Nipbl* loss sensitizes tumour cells to anti-PD-1 therapy *in vivo* independent of tumour neoantigen.**

Herein, we examined whether *Nipbl*-deficient tumours were sensitive to anti-PD-1 therapy. As shown in Fig. 3A, *Nipbl*-deficient colon26 tumours were almost completely regressed 40 days after anti-PD-1 treatment, and only 3 out of 12 tumours rebounded after treatment stopped. The survival rate of host mice bearing sgNipbl colon26 tumours was significantly improved after anti-PD-1 therapy compared to that of the vehicle group mice ($p < 0.0001$, Fig. 3B). In contrast, anti-PD-1 therapy had little or no effect on tumour growth and the survival rate of mice bearing sgControl colon26 tumours compared to the vehicle group mice ($p > 0.05$). Notably, anti-PD-1 therapy potently rejuvenated the CD8 TIL subpopulation compared to that of the vehicle group, as evidenced by increased Ki-67 and granzyme B expression levels ($p < 0.01$, Figs. 3C and S4B). We obtained similar results in a CT26 murine syngeneic colorectal tumour model. As shown in Fig. 3D and 3E, 19 out of 28 *Nipbl*-deficient CT26 tumours in mice completely disappeared after anti-PD-1 treatment; the survival rate of host mice bearing sgNipbl tumours was significantly prolonged compared to that of the vehicle group mice; In contrast, anti-PD-1 treatment had a weak inhibitory effect on sgControl CT26 tumours. Our data suggest that the PD-1/PD-L1 inhibitory checkpoint pathway is critical for maintaining the *in vivo* growth of *Nipbl*-deficient tumours.

228 Next, we asked whether anti-PD-1 therapy elicits an antitumour memory response
229 in the host (Fig. 3F). Tumour re-challenge data showed that *Nipbl*-deficient colon26
230 tumours were no longer able to grow in mice with completely regressed tumours even
231 124 days after tumour cell re-injection, but they developed quickly in age-matched
232 naïve mice (Fig. 3G). The survival rate of these tumour-free mice was significantly
233 prolonged compared to that of the naïve mice ($p < 0.0001$, Fig. 3H). We obtained
234 similar results in the CT26 mouse model (Fig. 3I and 3J). Flow cytometry analysis
235 revealed that the proportions of both CD8⁺ and CD4⁺ central memory T cells were
236 significantly increased in the spleens of tumour-free mice compared to those of naïve
237 mice ($p < 0.05$, Figs. 3K and S6), suggesting that anti-PD-1 therapy elicits antitumour
238 immune memory responses in the host.

239 Because NIPBL is critical for maintaining genome instability³², *NIPBL*-defective
240 tumours potentially increase their sensitivity to immunotherapy by producing
241 neoantigens. To exclude this possibility, we individually transplanted *Nipbl* wild-type
242 colon26 and CT26 parental tumours into sgNipbl tumour-free mice and observed that
243 these tumours were again fully rejected compared to those of age-matched naïve mice,
244 showing a significantly improved survival rate in the host (Fig. 3L and 3M),
245 suggesting that *Nipbl* loss did not affect tumour cell immunity by introducing
246 neoantigens in our case.

247 ***NIPBL* loss stimulates ERV expression in tumour cells by blocking DNMT1**
248 **transcription.**

249 To explore the mechanism by which *Nipbl* loss induces PD-L1 expression in

250 tumours, we profiled the transcriptome of HCT-116 and CT26 cell lines with
 251 sgNIPBL, sgSTAG2, shSMC1A and their counterparts (Fig. 4A). The knockout and
 252 knockdown efficiencies of these subclones were shown in Fig. S3. We obtained 71
 253 upregulated and 7 downregulated genes that overlapped in both HCT-116 and CT26
 254 cell lines (average fold > 1.5). Metascape analysis⁴³ revealed that genes related to type
 255 I/II interferon responses (GO: 0060337/0034341) and the host defence response to
 256 virus (R-HAS-1169410, hsa05168) were robustly clustered when comparing the
 257 cohesin loss and control groups ($p < 0.01$, Fig. 4B). Protein-protein interaction analysis
 258 also revealed that the host defence response to virus network (GO: 0051607)
 259 including IRF9, BST2, IFIT1, RSAD2, IFIT3, OAS3 and STAT2 was significantly
 260 enriched when cohesin genes were silenced (in orange, Fig. 4A and 4C). Moreover,
 261 genes related to cellular response to exogenous dsRNA (GO: 0071360), such as
 262 DDX58/RIG-I, IFIH1/MDA5 and LGALS9, were highly enriched in the top 30
 263 overlapping genes when comparing the sgNIPBL and sgControl groups (in red, Fig.
 264 4A and 4B). Genes related to dsRNA binding (GO: 0003725), such as RIG-I, MDA5
 265 and OAS3, were also enriched (Fig. 4A). As a positive control, the mRNA expression
 266 levels of PD-L1 (encoded by the CD274 gene, in blue) were significantly increased in
 267 all the tested samples. This observation led us to investigate whether dsRNA ERVs are
 268 actively expressed in *NIPBL*-deficient tumour cells.

269 Immunofluorescent staining data showed that dsRNAs were dramatically increased
 270 in the cytosol of sgNIPBL HCT-116 cells, accounting for approximately 75% of total
 271 cells but were limited to the nucleus of sgControl cells (Fig. 4D). The dsRNA content

in sgNIPBL HCT-116 cells was increased two-to-threefold compared to that of sgControl cells (Fig. 4E). Similarly, cytosolic dsRNAs were clearly observed at a higher level in sgNipbl CT26 cells compared to sgControl cells, accounting for approximately 75-90% of the total cells (Fig. 4F). The dsRNA content in the cytosol of sgNipbl CT26 cells was increased twofold compared to that of sgControl cells (Fig. 4G). dsRNAs were also clearly observed in the cytosol of endogenous *NIPBL*-deficient RKO and cloudman S91 tumour cell lines compared to wild-type HCT-116 and CT26 cells (Fig. 4H and 4I). The qRT-PCR data confirmed that human endogenous retroviruses (HERVs) such as HERV-E, HERV-F and HERV-K were significantly enhanced in sgNIPBL HCT-116 cells compared to sgControl cells (Fig. 4J). Based on these results, we conclude that *NIPBL* loss induces ERV expression in tumour cells.

ERVs are DNA sequences of retroviral origin that integrate into the mammalian genome, accounting for approximately 8-10% of the human and mouse genomes^{44, 45}. The transcription of ERVs is strictly repressed in somatic cells by DNMT1-mediated DNA methylation to avoid the activation of host antiviral innate immunity^{46, 47}. Thus, we asked whether *NIPBL* loss would affect DNMT1 expression. Expectedly, DNMT1 was markedly decreased at both the mRNA and protein levels when *NIPBL* was knocked out in HCT-116 cells (Fig. 4K). DNMT1 protein expression was also significantly reduced in three endogenous *NIPBL*-mutant human colorectal cancer cell lines (Fig. 4L). We did not observe obvious changes in either CDK4 or CDK6 protein levels in this case⁴⁸. Next, we repeated this experiment using DNMT1 inhibitor

294 5'-azacytidine⁴⁹. Compared to the vehicle, 5'-azacytidine robustly induced PD-L1
295 expression at both the mRNA and protein levels in HCT-116 cells (Fig. 4M). In
296 contrast, the Myc inhibitor JQ1 had little or no influence on PD-L1 expression. This
297 suggests that NIPBL loss-induced DNMT1 inhibition but not Myc inhibition is critical
298 for inducing PD-L1 expression in tumour cells⁵⁰.

299 Noteworthy, anti-PD-1 therapy significantly prolonged overall survival of
300 colorectal cancer and melanoma patients harbouring mutations in *STAG2*, *RAD21* and
301 *DNMT1* compared to that of their counterparts without mutations (colorectal cancer:
302 p=0.043; melanoma: p=0.039, Fig. 4N, 4O and Supplemental Table 1)⁵¹. Because only
303 the genetic mutations of *STAG2* and *RAD21* but no other members of cohesin core
304 subunits and regulators are assessable in these Memorial Sloan Kettering Cancer
305 Center (MSKCC) immunogenomic studies⁵¹, we included DNMT1 mutation based on
306 the mechanism of cohesin loss-induced DNMT1 inhibition.

307 We next asked whether NIPBL directly regulates DNMT1 transcription. By
308 assessing the ENCODE data⁵², we identified that NIPBL may interact with the
309 DNMT1 promoter. The chromatin immunoprecipitation (ChIP)-PCR data showed that
310 NIPBL selectively bound with the DNMT1 promoter in the -481-bp to +121-bp region
311 compared to that of the control primer (Fig. 4P). We obtained similar results using
312 IgG as a negative control. There were two potential NIPBL binding sites located in the
313 -481-bp to +121-bp region of the DNMT1 promoter (Fig. 4P, left)⁵². Thus, we
314 individually cloned the DNMT1 promoter (-481 bp to +121 bp and -269 bp to +121
315 bp) into a pGL3-basic luciferase vector and observed that the luciferase activity of the

316 DNMT1 promoter (-481 bp to +121 bp) was greatly impaired when binding site 2
317 (-481 bp to -370 bp) was removed (Fig. 4Q, left). The luciferase activity of the
318 DNMT1 promoter (-481 bp to +121 bp) was significantly reduced in sgNIPBL
319 HCT-116 cells compared to sgControl cells. Similarly, the luciferase activity of the
320 DNMT1 promoter (-481 bp to +121 bp) was also impaired in *NIPBL*-deficient RKO
321 cells compared to wild-type HCT-116 cells (Fig. 4Q, right). This suggests that the
322 binding site 2 is critical for NIPBL-mediated DNMT1 transcription.

323 Collectively, we conclude that *NIPBL* loss provokes ERV expression by impairing
324 DNMT1 transcription.

325 **The dsRNA-RIG-I/MDA5-MAVS-IRF3 signalling is responsible for *NIPBL***
326 **loss-induced PD-L1 expression.**

327 The RIG-I/MDA5-MAVS signalling pathway is responsible for detecting cytosolic
328 viral RNA⁵³⁻⁵⁸. Immunoblotting data showed that the core members of the
329 RIG-I/MDA5-MAVS signalling machinery, such as RIG-I, MDA5, MAVS, IRF3 and
330 IRF7, were robustly increased when *NIPBL* was knocked out in HCT-116 cells (Fig.
331 5A). We also observed that STAT1, STAT2 and IRF9, which are key regulators for
332 type I/II interferon responses⁵⁹, were highly expressed in sgNIPBL HCT-116 tumour
333 cells compared to sgControl cells. Similar results were obtained in *NIPBL*-mutant
334 CW2 and LoVo colorectal tumour cell lines, which showed increased PD-L1
335 expression compared to that in control cells (Fig. 5B). To investigate whether
336 RIG-I/MDA5-MAVS signalling is required for *NIPBL* loss-induced PD-L1 expression,
337 we silenced MAVS using three different shRNAs and observed that shMAVS

338 obviously reduced PD-L1 expression in sgNIPBL HCT-116 cells compared to
339 scramble cells (Fig. 5C). MAVS knockdown also greatly reduced PD-L1 expression in
340 *NIPBL*-mutant RKO tumour cells (Fig. 5D). Notably, exogenous overexpression of
341 wild-type MAVS recaptured the transcriptional signature of the sgNIPBL group,
342 which (compared to the control group) showed increased mRNA and protein
343 expression of genes related to the defence response to virus (GO: 0051607), such as
344 RIG-I, MDA5, STAT1, STAT2 and IRF9 plus CD274 (Fig. 5E and 5F). We did not
345 observe a significant change in IRF3 protein due to the large amount of IRF3 protein
346 in 293T cells, but the phosphorylation of IRF3 at serine 386 was increased. This
347 suggests that the dsRNA-MAVS signalling is crucial for the *NIPBL* loss-induced cell
348 phenotype.

349 IRF3 and IRF7 are two major downstream effectors of the dsRNA-MAVS
350 signalling^{60, 61}, and both showed increased protein expression levels in
351 *NIPBL*-deficient tumour cells compared to control cells (Fig. 5A and 5B). Thus, we
352 assessed whether IRF3 or IRF7 is critical for *NIPBL* loss-induced PD-L1 expression.
353 shRNA silencing data demonstrated that IRF3 knockdown abrogated PD-L1 protein
354 expression in *NIPBL*-deficient HCT-116 and RKO tumour cells (Fig. 5G). In contrast,
355 IRF7 knockdown had little or no effect on PD-L1 expression (Fig. 5H). These data
356 suggest that the MAVS-IRF3 signalling is critical for *NIPBL* loss-induced PD-L1
357 expression.

358 In the literature, the activation of MAVS-IRF3 signalling often leads to the
359 production of IFN- β in non-immune cells, which is critical for antiviral innate

immunity⁵⁴⁻⁵⁸. The qRT-PCR data revealed that the mRNA expression levels of IFN- α ,
IFN- β and IFN- γ , but not type III interferons IL-28 and IL-29, were low or
undetectable in *NIPBL*-deficient tumour cells (Fig. 5I), which is consistent with
previous reports^{61, 62}. We also detected no phosphorylation of STAT2 at Tyr 690 in
sgNIPBL tumour cells, which is rapidly activated by JAK upon IFNs stimulation⁵⁹.
We further treated sgNIPBL HCT-116 cells with two JAK inhibitors and observed that
both AZD1480 and CYT387 had no significant effect on PD-L1 protein expression by
comparing sgNIPBL HCT-116 cells and sgControl cells, although STAT1 Y701
phosphorylation was largely abolished (Fig. 5J). In addition, we individually
incubated sgControl HCT-116 and colon26 cells with sgNIPBL tumour cell medium
for 48 hours and observed no change in PD-L1 protein expression (Fig. S7). These
data suggest that NIPBL loss-induced PD-L1 expression is not IFNs-dependent.

Using dsRNA synthetic analog polyinosinic-polycytidylic acid (poly (I:C)) as a
positive stimuli, we observed that IFN- β but not IFN- α/γ was robustly increased
approximately 4000-fold in sgNIPBL HCT-116 cells compared to that of sgControl
group (Fig. 5K). Because the IFN- β transcription is co-ordinately regulated by the
IFN- β enhanceosome consisting of IRF3/7, NF- κ B and phosphorylated JNK, MEK
and p38-mediated AP-1 activation^{63, 64}, we profiled these protein expression levels
and observed that nuclear NF- κ B and phosphorylated JNK were weakly expressed in
sgNIPBL HCT-116 cells, but were highly activated by poly (I:C) treatment compared
to that of vehicle (Fig. 5L). p38 and MEK remained highly phosphorylated in all the
cases. To examine whether NF- κ B or JNK activation is crucial for IFN- β expression,

we individually treated sgNIPBL HCT-116 cells with small molecular inhibitors of NF- κ B (BAY 11-7082 and pyrrolidinedithiocarbamate ammonium (PDTC)) or JNK (SP600125) and observed that two NF- κ B inhibitors but not JNK inhibitor dramatically reduced approximately 75-95% of poly (I:C)-induced IFN- β expression in sgNIPBL HCT-116 cells (Fig. 5M), suggesting that NF- κ B activation is essential for IFN- β expression. Noteworthy, NIPBL-induced MAVS-IRF3/7 activation is also crucial for IFN- β expression because sgControl HCT-116 tumour cells with lowly expressed IRF3/7 had a very weak IFN- β expression upon poly (I:C) treatment. Taken together, we conclude that NIPBL loss-induced ERV expression selectively increase PD-L1 expression but not IFN- β due to lack of NF- κ B activation.

IRF3 stimulates PD-L1 expression by assembling the STAT2-IRF9 complex.

To further explore the mechanism of PD-L1 regulation, we cloned the PD-L1 promoter into a pGL3-basic vector and observed that the luciferase activity of the PD-L1 promoter (the -799-bp to +153-bp region from the translational starting site) was 50-fold higher than that of the vector control (Fig. 6A), suggesting that this promoter region is critical for PD-L1 transcription. Promoter truncation analysis revealed that the luciferase activity of the PD-L1 promoter was reduced by 80% in sgNIPBL HCT-116 cells when the -223-bp to -133-bp region was removed (Fig. 6B). We obtained similar results in *NIPBL*-mutant RKO cells (Fig. 6C). This suggests that the -223-bp to -133-bp region of the PD-L1 promoter is responsible for *NIPBL* loss-induced PD-L1 expression. DNA binding consensus motif analysis revealed that one STAT2 and one IRF binding site were potentially located in this small region (Fig.

6A). As previously mentioned, STAT2, IRF3, IRF7 and IRF9 were markedly increased in NIPBL-deficient tumour cells compared to control cells, while IRF1 was weakly or not expressed (Figs. 5A, 5B and 6D). Thus, we asked whether IRFs or its combination with STAT2 could induce PD-L1 expression. Using IRF1 as a positive control⁶⁵, exogenous overexpression of IRF1, IRF1 plus STAT2 and STAT2 plus IRF9 robustly enhanced the luciferase activity of the 386-bp PD-L1 promoter, while STAT2 alone, other IRFs or their combination with STAT2 failed to do so (Fig. 6E). Remarkably, individual knockout of STAT2 and IRF9 obviously abrogated PD-L1 expression in sgNIPBL HCT-116 cells compared to that in the control cells (Fig. 6F). In contrast, IRF1 knockdown had little or no influence on PD-L1 expression (Fig. 6G). Knockdown of STAT1, which was located outside of the -223-bp to -133-bp region, also had no effect on PD-L1 expression (Fig. 6H). The analysis of TCGA tumour data revealed that both STAT2 and IRF9 well correlated with PD-L1 expression at mRNA levels in stomach and colorectal tumour samples (Figs. 6I and S8). These results indicate that STAT2 and IRF9 are potentially responsible for NIPBL loss-induced PD-L1 expression.

Next, we asked whether co-expression of STAT2 and IRF9 induces PD-L1 expression. As expected, STAT2 and IRF9 robustly increased PD-L1 at both the mRNA and protein levels, accompanied by elevated STAT1 expression (Fig. 6J and 6K). To further exclude STAT1 and IRF1, we individually established sgSTAT1 and sgIRF1 293T clones and observed that co-expression of STAT2 and IRF9 robustly increased PD-L1 expression in these cells (Fig. 6L), suggesting that STAT2 and IRF9

co-ordinately regulate PD-L1 transcription independent of IRF1 and STAT1. In the literature, STAT2 but not STAT1 is evolutionarily conserved to interact with IRF9⁶⁶. Thus, we performed an immunoprecipitation (IP) assay and observed that STAT2 physically associated with IRF9 in sgNIPBL HCT-116 cells (Fig. 6M). Using a reciprocal IP assay, we observed that IRF9 selectively binds with STAT2 but not STAT1 (Fig. 6N). Dual immunofluorescent staining data illustrated that STAT2 and IRF9 were largely co-localized in the nucleus of sgNIPBL HCT-116 cells (Fig. 6O).

The ChIP-PCR data confirmed that STAT2 and IRF9 co-occupied the PD-L1 promoter close to the -208-bp to -148-bp regions upstream of the translation start site (Fig. 6A and 6P). Luciferase expression data showed that co-expression of STAT2 and IRF9 increased PD-L1 promoter activity by approximately 11-fold, but this effect was completely abolished when the 60-bp STAT2-IRF9 DNA binding motif was removed (Fig. 6A and 6Q). We cloned this 60-bp DNA fragment and observed that it responded to the co-expression of STAT2 and IRF9 (Fig. 6R). Thus, we conclude that the STAT2-IRF9 complex is essential for *NIPBL* loss-induced PD-L1 expression in tumour cells.

Finally, we asked whether IRF3 is responsible for STAT2 and IRF9 expression. We generated a constitutive active IRF3 5D mutant by substituting Ser396, Ser398, Ser402, Thr404 and Ser405 with aspartic acids⁶⁷ and observed that exogenously expression of IRF3 5D mutant but not wild-type robustly activated STAT2, IRF9 and PD-L1 at both mRNA and protein levels (Fig. 6S). Consistently, IRF3 5D mutant significantly increased the luciferase expression levels of IRF9 and STAT2 promoters

448 containing the AANNGAAA motif compared to that of wild-type IRF3 (Fig. 6T).

449 Meanwhile, shIRF3 markedly reduced the mRNA expression levels of both STAT2

450 and IRF9 in sgNIPBL HCT-116 cells compared to scramble (Fig. 6U). These results

451 indicate that IRF3 promotes PD-L1 transcription in NIPBL-deficient tumours by

452 transcriptionally upregulating the STAT2-IRF9 complex.

453

454 Discussion

455 ERV expression, which often leads to immunosuppression in the host⁶⁸, has
 456 recently been identified in numerous human cancer types and is well correlated with
 457 patient responses to immunotherapy^{69, 70}. Our study reveals that ERV expression is a
 458 consequence of cohesin loss-mediated DNMT1 inhibition in solid tumour cells and
 459 inactivates host CD8 TILs via the PD-L1/PD-1 inhibitory checkpoint pathway.
 460 Mechanistically, ERV expression selectively promotes the PD-L1 expression by
 461 stimulating the dsRNA-MAVS-IRF3-STAT2/IRF9 signalling pathway, but doesn't
 462 induce IFN- β expression due to lack of NF- κ B activation. *Villin*-driven *Nipbl*
 463 deficiency significantly promotes tumour growth in the host when crossed with
 464 *APC^{min}* mice, reinforcing that *Nipbl*-deficient tumours are immunosuppressive.
 465 Noteworthy, cohesin loss leads to defective interferon responses in human acute
 466 myeloid leukaemia, of which high STAT1 and STAT2 expression levels are critical for
 467 the development and antiviral and antitumour immunity of immune cells⁷¹⁻⁷⁴.
 468 Nevertheless, our study provide a novel mechanism by which tumour-associated
 469 ERVs establish an immunosuppressive microenvironment.

470 Although DNMT1 inhibitors are clinically used for treating lymphomas by
 471 producing viral mimicry responses^{61, 62}, mice with highly reduced Dnmt1 expression
 472 levels spontaneously developed T cell lymphoma⁴⁷. The TCGA data analysis revealed
 473 that patients with low DNMT1 mRNA expression had a significantly worse survival
 474 rate than those with high DNMT1 expression in both stomach and colorectal tumour
 475 cohorts (Fig. S9). This observation led us to investigate whether PD-L1 is critical for

the relapse of low DNMT1-expressing tumours. To verify this, we pretreated lewis lung cancer cells with 0.5 μ M decitabine for 3 days and inoculated them into the flanks of syngeneic mice and observed that anti-PD-1 monoclonal antibody potently reduced the *in vivo* growth of decitabine-pretreated lewis lung cancer cells compared to that with vehicle, and 2 out of 3 tumours completely regressed ($p=0.011$, Fig. S10A). PD-L1 protein expression was visibly increased after treatment with decitabine (Fig. S10B). As a control, lewis lung cancer cells showed no significant difference between the vehicle and anti-PD-1 groups ($p>0.05$, Fig. S10C). Consistently with previous report⁷⁵, the PD-L1 expression was often increased after DNMT1 inhibitor treatment (Fig. 4M). Thus, it would be ideal to combine with anti-PD-1 therapy when applying DNMT1 inhibitors to patients when it elicits PD-L1 expression in tumour cells.

In the literature, it is generally accepted that a high mutation burden in tumours is associated with the clinical outcomes of anti-PD-1 therapy^{31, 51, 76, 77}. Changes leading to deficiency of DNA mismatch repair, such as *Mlh1* loss, can increase the mutational burden of tumour cells and increase tumour sensitivity to immune checkpoint inhibitors^{4, 78}. To assess whether mutations of cohesin subunits and regulators are correlated with high mutation load, we analysed MSKCC immunogenomic data and observed that mutations of STAG2, RAD21 and DNMT1 were well correlated with a higher mutation load in MSI-subtype colorectal cancer samples ($n=109$, $p=4.42E-5$, Supplemental Table 1); however, mutations of STAG2, RAD21 and DNMT1 were poorly associated with a high tumour mutation load in microsatellite stable (MSS)

498 melanoma (n=245, p=0.92, Supplemental Table 1). The average mutation counts for
499 melanoma and colorectal cancer were 773.1 and 27.7, respectively.

500 Because *NIPBL* is frequently mutated at repetitive DNA sequence sites, which is a
501 characteristic of tumours with MSI⁷⁹⁻⁸¹, we further generated *Mlh1* knockout CT26
502 cells and performed another round of GeCKO screening, showing that sgRNAs
503 targeting cohesin subunits (*Stag1*, *Smc1b* and *Rad21*) and regulators (*Nipbl*, *Pds5a*,
504 *Pds5b* and *Esco1*) were again selected out in the anti-PD-1/vehicle group compared to
505 nontargeting sgRNA group (p<0.005, Fig. S11). As shown in Fig. 2E, murine B16F10
506 melanoma cells used in previous CRISPR-Cas9 library screens are *Nipbl*-deficient,
507 which explains why cohesin genes were not profiled²². Notably, *Nipbl* depletion in
508 gastrointestinal tissue is not sufficient to elicit tumour in mice, showing a normal
509 lifespan and pregnancy in the host, suggesting that other cancer driver mutations are
510 required to promote tumour formation *in vivo*. These results indicate that cohesin
511 loss-induced tumour immunity is likely independent of a high tumour mutation load
512 but has a greater chance of occurring when there is a high number of gene mutations.

513 As core components of the transcriptional machinery, DNA polymerases such as
514 POLE and POLD1 are highly mutated in human cancer and are well associated with a
515 high tumour mutation burden³¹. Thus, it is particularly of interest to explore whether
516 DNMT1 inhibition commonly co-occurs with the deregulation of transcriptional
517 machinery, including cohesin, mediators and DNA polymerases. If that were the case,
518 low DNMT1 expression or ERV expression could serve as an independent biomarker
519 for predicting the therapeutic responses to anti-PD-1 therapy.

520 In the literature, IFN- γ is well-known for boosting PD-L1 expression in tumour
 521 cells through STAT1-IRF1 signalling^{65, 82, 83}. We observed little or no IFN- γ and IRF1
 522 expression in *NIPBL*-deficient tumour cells, but STAT1 was actively expressed when
 523 ERV or IRF3 5D mutant was expressed (Figs. 5A and 6S). Individual knockdown of
 524 IRF1 and STAT1 did not affect PD-L1 expression in *NIPBL*-deficient tumour cells.
 525 JAK inhibitor also didn't affect PD-L1 expression, although it potently blocked
 526 STAT1 phosphorylation at Tyr701 site. These results suggest that the STAT1-IRF1
 527 signalling is dispensable for ERV-induced PD-L1 expression. Interestingly, depletion
 528 of STAT2 and IRF9 had also no impact on IFN- γ -induced PD-L1 expression
 529 compared to that with STAT1 knockout (Fig. S12). These results indicate that the
 530 STAT2-IRF9 complex plays a unique role in ERV-induced PD-L1 expression, which
 531 is separate from IFN- γ -induced PD-L1 expression via the STAT1-IRF1 complex.

532 In summary, our study reveals a novel mechanism by which *NIPBL* deficiency
 533 regulates immunity in cancer by stimulating ERV expression; *NIPBL*-deficient
 534 tumours are highly immunosuppressive, but this effect could be rejuvenated by
 535 anti-PD-1 therapy.

536

537 **Material and methods**

538 **Cell lines.** CT26, Lewis lung cancer, B16F10, HEK-293T, CW2, LoVo, RKO and
 539 HCT-116 cell lines were purchased from the Cell Bank of Shanghai Institutes for
 540 Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Colon26 and
 541 cloudman S91 murine cell line was purchased from BeNa Culture Collection (Beijing,
 542 China). All cells were maintained in DMEM/F12 or RPMI 1640 medium
 543 supplemented with 10% fetal bovine serum and 1% penicillin streptomycin and
 544 incubated at 37°C in a humidified incubator with 5% CO₂. All human cell lines were
 545 authenticated by STR. The strain source of mouse cell lines was identified using
 546 PCR⁸⁴. CT26 and colon26 were subjected to exon sequencing. All cell lines were
 547 tested negative for mycoplasma contamination.

548 ***In vivo* mouse GeCKO screening.** The GeCKO screening was performed following
 549 previously reports^{19, 85, 86}. CT26 cells were infected with lentiviruses containing
 550 GeCKO library sgRNAs at a multiplicity of infection (MOI) of 0.3, and were selected
 551 with puromycin for 7 days to remove uninfected cells. After selection, 2×10⁷ cells
 552 were directly harvested as DMSO_{Day0} group and frozen for genomic DNA extraction,
 553 and the remaining cells were inoculated into the flanks of syngeneic mouse for
 554 receiving *in vivo* screening (2×10⁷ cells per injection, 10 mice per group). Mice were
 555 randomly assigned to two groups treated with control IgG or anti-PD-1 monoclonal
 556 antibody (ip. 200 µg/mouse, clone RMP1-14, Bio X cell) at day 0, 3, 5, 8, 11 post
 557 inoculation, respectively.

558 After 21 days post inoculation, the genomic DNA was individually extracted from

559 tumour tissues of each mouse and was amplified for Illumina sequencing using
560 two-step PCR⁵⁰. The second PCR products were purified using Qiagen gel extraction
561 kit. To avoid tumour size imbalance, equal amounts of PCR products of each tumour
562 sample was picked up and mixed in each group for Illumina HiSeq X10 sequencing
563 by Novogen according to the manufacturer's instruction. The sgRNA averages of
564 DMSO_{day0}, Control_{day21} and anti-PD-1_{day21} groups were 487, 404 and 516, respectively,
565 which was approximately 400-fold of total library sgRNAs. The library coverage rates
566 of DMSO_{day0}, Control_{day21} and anti-PD-1_{day21} groups were 99.35% (65531/65959
567 sgRNAs), 68.06% (44894/65959 sgRNAs) and 89.68% (59156/65959 sgRNAs),
568 respectively. The loss of library sgRNAs in the Control_{day21} group is likely caused by
569 tumour overgrowth.

570 After normalized with medium normalization, P-values of sgRNAs were
571 calculated using a MAGeCK algorithm that test whether sgRNA abundance differs
572 significantly between control IgG and anti-PD-1 groups. sgRNAs were then ranked
573 based on P-values using a modified robust ranking aggregation algorithm to identify
574 positively or negatively selected genes²⁴. The fold changes of sgRNAs were
575 calculated by comparing the read counts of anti-PD-1 group to that of control IgG
576 group after normalized. Similar experimental procedure was applied in *Mlh1*
577 knockout CT26 tumour screening.

578 ***In vivo* anti-PD-1 treatment.** All the mice were purchased from Shanghai SLAC
579 Laboratory Animal Co. Ltd. All studies were performed according to the guidelines of
580 Institutional Animal Care and Use Committee of Shanghai Institute of Nutrition and

581 Health. For xenograft tumours, 5×10^5 CT26 or 1×10^6 colon26 cells were
582 subcutaneously injected into the flanks of the 6-week-old syngeneic BALB/C mice.
583 After injected, mice were randomly assigned to different groups, followed by the
584 treatment of vehicle or anti-PD-1 antibody (i.p., 200 μ g/mouse, Bio X cell, clone
585 RMP1-14) at day 0, 3, 5, 8, 11 post inoculation. Tumour size was measured twice one
586 week, and tumour volume was determined based on the following formula: Tumour
587 volume = $0.5 \times \text{length} \times \text{width}^2$.

588 **Statistics.** All statistical analyses were done in GraphPad Prism 7 software. Survival
589 significance was assessed by a Logrank Mantel-Cox test. All data were presented as
590 means $\pm$ SD. The p value was calculated by two-tailed *t* tests. *, $p < 0.05$, **, $p < 0.01$,
591 ***, $p < 0.001$.

592 **Data Availability.** All the datasets are available from the corresponding author upon
593 reasonable request. sgRNA library screening sequencing data and RNA-Seq data have
594 been deposited into NCBI database under accession number GSE125201, GSE125202
595 and GSE124909.

596 Please see more information in supplemental data.

597

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604

605 **Author contributions**

606 J.Y.L conceived and supervised the study. J.Y.L and Y.H. wrote the manuscript. Y.H.

607 performed the experiments on sgRNA library screening, Nipbl knockout cell line, in

608 vivo anti-PD-1 therapy and STAT2-IRF9 function. F. P. performed the dsRNA- and

609 DNMT1-related experiments. Y.C. performed the experiments on the MAVS-IRF3

610 signalling and Nipbl knockout mice. T. L. performed experiments on NIPBL^{fl/fl};

611 APC^{Min} mice, Nipbl knockout cell line and statistical analysis on mutations of cohesin

612 subunits and regulators in TCGA samples. J.S. performed Nipbl wild-type tumour

613 re-challenge experiments. Z.C. performed the statistical analysis of melanoma and

614 colorectal cancer patients responding to anti-PD-1 therapy. Q.D helped on flow

615 cytometric analysis of tumour infiltrated immune cells. F.J., H.X., H.Z., X.K., Q.C.,

616 J.D. contributed to discussion of results.

617

618

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801

802 **Figure legends**

803 **Figure 1. *In vivo* GeCKO screening reveals that loss of cohesin subunits and**
804 **regulators sensitizes tumour cells to anti-PD-1 therapy.** (A) Schematic diagram of
805 the *in vivo* GeCKO screening strategy. (B) Compared to non-targeting sgRNAs,
806 sgRNAs targeting cohesin subunits and regulators were significantly reduced by
807 comparing the anti-PD-1 group to control IgG group. The dashed lines indicate a p
808 value of 0.005. Annotated genes represent cohesin subunits and regulators (red),
809 SWI/SNF complex (blue) and glycolysis (green) genes validated by other groups, and
810 genes with p values > 0.005 (grey). (C) The mutation frequencies of 13 cohesin
811 subunits and regulators in the pan-cancer atlas samples (n=10967). Cartoon of cohesin
812 complex subunits and regulators. (D) Mutations of 13 cohesin subunits and regulators
813 negatively correlated with the patient's overall survival of pan-cancer samples. (E)
814 The distribution of inactive mutations and null expression ($\log_2(\text{RPKM}) < -2$) of the
815 indicated cohesin subunits and regulators in PD-L1-positive ($\log_2(\text{RPKM}) > 0.5$)
816 tumour samples of patients across 18 cancer types from the TCGA database. (F)
817 Mutations in *NIPBL*, *STAG2*, *STAG3* and *SMC1B* significantly prolonged the
818 progression-free survival of lung cancer patients treated with pembrolizumab (n=5)
819 compared with patients with non-cohesin complex member mutations (n=25)
820 (log-rank p=0.012). The survival significance was assessed by the log-rank
821 Mantel-Cox test.

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Figure 2. Nipbl loss suppresses tumour infiltrated CD8+ T cells by inducing the PD-L1/PD-1 inhibitory checkpoint pathway. (A) Flow cytometric analysis of PD-L1 protein expression in Nipbl knockout CT26 and colon26 syngeneic tumour models compared to sgControl models. The statistical bar diagram is shown on the bottom. (B-D) The indicated protein expression levels were detected by immunoblotting of CT26, colon26 and HCT-116 tumour cell lines, while the mRNA expression levels were determined by qRT-PCR. (E) PD-L1 and Nipbl protein expression was detected by immunoblotting in murine B16F10 and cloudman S91 tumour cell lines. (F) PD-L1 and NIPBL protein expression was detected in the human NIPBL^{p.K603fs*11} RKO colorectal tumour cell line. (G) Correlation of NIPBL mRNA levels in tumour cells with the PD-1 mRNA levels in infiltrating immune cells of TCGA colorectal cancer patient samples (n=457). Spearman's correlation and estimated significance were calculated with the Tumor Immune Estimation Resource (TIMER) and adjusted for tumour purity. (H) Analysis of immune cell infiltration in NIPBL wild-type and NIPBL-mutant TCGA colorectal cancer samples (n=457). (I-J) The percentages of CD45+ cells and CD8+ TILs were quantitated (CT26 tumours: sgControl, n=18, sgNipbl#1, n=15, sgNipbl#2, n=9; colon26 tumours: sgControl, n=10, sgNipbl#1, n=13, sgNipbl#2, n=6). (K) The percentages of CD8+ and CD4+ TILs, Treg cells, macrophage, myeloid-derived suppressor cells (MDSCs) and natural killer (NK) cells were quantitated, normalized by CD45+ cells. (L) Representative plots showing the gating for the exhausted CD8+ T cell subpopulation characterized by PD-1+ and EOMES+ staining. (M) The percentages of exhausted CD8+ T cells in

846 the indicated groups were quantitated (CT26 tumours: sgControl, n=18, sgNipbl#1,
847 n=15, sgNipbl#2, n=9; colon26 tumours: sgControl, n=10, sgNipbl#1, n=13,
848 sgNipbl#2, n=6). (N) The survival rates of Nipbl^{fl/fl} mice (n=5), Villin-Cre⁺; Nipbl^{fl/fl}
849 mice (n=7) and Villin-Cre⁺; Nipbl^{fl/fl} mice (n=4). For the loxP site in intron 4 of the
850 Nipbl^{fl/fl} allele, the PCR products were 300 bp for the floxed allele and 330 bp for the
851 wild-type allele. (O) The survival rates of APC^{min} mice (n=17) and Villin-Cre⁺;
852 Nipbl^{fl/fl} mice; APC^{min} (n=6). The expression levels of the indicated proteins were
853 determined by immunoblotting. The survival significance was assessed by the
854 log-rank Mantel-Cox test. Data are shown as the mean $\pm$ SD, and the p value was
855 calculated by two-tailed t tests.*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
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Figure 3. Nipbl-deficient tumours are supersensitive to anti-PD-1 therapy *in vivo*, eliciting antitumour memory responses in the host. (A-B) The therapeutic responses and survival rates of syngeneic mice bearing sgControl or sgNipbl colon26 tumours receiving anti-PD-1 mAb or vehicle treatment (sgControl group: vehicle, n=10; anti-PD-1, n=10; sgNipbl group: vehicle, n=10; anti-PD-1, n=12). (C) The percentages of reactive cytotoxic CD8⁺ T cells showing Ki-67⁺ and granzyme B⁺ expression in sgNipbl colon26 tumours after vehicle or PD-1 mAb treatment were quantitated (vehicle, n=4; anti-PD-1, n=7). Representative plots showing the gating for reactive CD8⁺ T cells showing Ki-67⁺ and granzyme B⁺ expression. (D-E) The therapeutic responses and survival rates of syngeneic mice bearing sgControl or sgNipbl CT26 tumours treated with anti-PD-1 mAb or vehicle (sgControl group: vehicle, n=18; anti-PD-1, n=18; sgNipbl group: vehicle, n=16; anti-PD-1, n=28). (F) Schematic diagram of the tumour re-challenge experiment. (G-H) Tumour growth and survival of tumour-free (n=8) and age-matched naïve mice (n=10) re-challenged with 1×10^6 sgNipbl colon26 tumours 79 days after the first round of anti-PD-1 therapy. (I-J) Tumour growth and survival of tumour-free (n=16) and age-matched naïve mice (n=8) re-challenged with 5×10^5 sgNipbl CT26 tumours 79 days after the first round of anti-PD-1 therapy. (K) The percentages of CD62L⁺/CD44⁺ central memory CD8⁺ T and CD4⁺ T cells in the spleens of tumour-free mice (colon26, n=9; CT26, n=8; age-matched naïve mice, n=7). (L) Tumour growth and survival of tumour-free (n=4) and age-matched naïve mice (n=10) re-challenged with 1×10^6 parental colon26 tumours post anti-PD-1 therapy. (M) Tumour growth and survival of tumour-free (n=8)

879 and age-matched naïve mice (n=8) re-challenged with 5×10^5 parental CT26 tumours
 880 post anti-PD-1 therapy. The significance of differences in survival was assessed by the
 881 log-rank Mantel-Cox test. Data are shown as the mean $\pm$ SD, and the p value was
 882 calculated by two-tailed t tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Figure 4. *NIPBL* loss stimulates ERV expression in tumour cells by blocking DNMT1 transcription. (A) The top 30 overlapping genes of the indicated groups were subjected to unbiased clustering by CLUSTER 3.0. (B) Representative enriched gene signatures by comparing the cohesin loss group to control groups. (C) IRF9-based protein interaction network was selected out by Metascape analysis. (D) dsRNA in HCT-116 cells was detected by a dsRNA-specific antibody using immunofluorescent staining. Bar diagram of cytosolic dsRNA-positive cells with statistics. (E) The dsRNA content was measured after removing DNA. (F) Immunofluorescent staining of dsRNA in sgControl and sgNipbl CT26 cells. Bar diagram of cytosolic dsRNA-positive cells with statistics. (G) The cytosolic dsRNA content was measured after removing DNA. (H-I) The cytosolic dsRNA of NIPBL-deficient RKO and cloudman S91 cells was imaged and quantified, while NIPBL wild-type HCT-116 and CT26 cells were used as controls. (J) The mRNA expression levels of HERV-E, HERV-F and HERV-K were determined by qRT-PCR. (K) The DNMT1 protein and mRNA expression levels were determined in sgControl and sgNIPBL HCT-116 cells. (L) The DNMT1, CDK4 and CDK6 protein levels were detected in three NIPBL-mutant colorectal tumour cell lines. (M) The protein and mRNA expression levels of the indicated genes were determined after treated with 2 μ M azacitidine or JQ1 for 36 hours. (N) Mutations in STAG2, RAD21 and DNMT1 significantly prolonged the overall survival of colorectal cancer patients treated with anti-PD-1 therapy (n=12) compared with that of non-mutation counterparts (n=97) (log-rank $p=0.043$). (O) Mutations in STAG2, RAD21 and DNMT1 significantly

907 prolonged the overall survival of melanoma patients treated with anti-PD-1 therapy
 908 (n=24) compared with that of non-mutation counterparts (n=221) (log-rank p=0.039).
 909 (P) ChIP-PCR analysis of NIPBL binding sites located in the DNMT1 promoter. (Q)
 910 The luciferase activity of the indicated DNMT1 promoter in HCT-116 sgControl,
 911 sgNIPBL and RKO cells. Data are shown as the mean $\pm$ SD, and the p value was
 912 calculated by two-tailed t tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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Figure 5. The dsRNA-MAVS-IRF3 signalling is responsible for *NIPBL* loss-induced PD-L1 expression. (A-B) The indicated proteins of the dsRNA-MAVS signalling machinery plus other proteins were detected by immunoblotting. (C-D) PD-L1 protein expression was detected by immunoblotting when MAVS was knocked down. (E-F) The mRNA and protein levels of the dsRNA-MAVS signalling machinery were detected when MAVS was exogenously overexpressed. (G) PD-L1 protein expression was detected in sgNIPBL HCT-116 and RKO cells by immunoblotting when IRF3 was knocked down. (H) PD-L1 protein expression was detected in sgNIPBL HCT-116 and RKO cells by immunoblotting when IRF7 was knocked down. (I) The mRNA expression levels of IFN- α , IFN- β , IFN- γ , IL-28 and IL-29 were determined in sgControl and sgNIPBL HCT-116 cells by qRT-PCR. (J) The indicated protein expression levels were determined by immunoblotting in the absence or presence of 0.5 μ M AZD1480 or CYT387. (K) The mRNA expression levels of IFN- α , IFN- β and IFN- γ were determined in sgControl and sgNIPBL HCT-116 cells in the absence or presence of 500 ng/ml poly (I:C) treatment. (L) Indicated proteins were detected by immunoblotting in the absence or presence of 500 ng/ml poly (I:C) treatment. (M) The IFN- β mRNA level was determined by qRT-PCR with or without NF- κ B inhibitor (BAY 11-7082 and PDTC) or JNK inhibitor (SP600125) treatment. Data are shown as the mean $\pm$ SD, and the p value was calculated by two-tailed t tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 6. STAT2 and IRF9 co-regulate PD-L1 transcription independent of STAT1 and IRF1. (A) Schematic diagram of the PD-L1 promoter. (B-C) The relative firefly luciferase expression levels of the indicated PD-L1 promoter in sgNIPBL HCT-116 and RKO cells. Renilla luciferase was used as an internal control. (D) The mRNA expression levels of the indicated genes in sgNIPBL HCT-116 cells were determined by qRT-PCR. GAPDH was used as an internal control. (E) The luciferase activity of the 386-bp PD-L1 promoter was measured when IRFs alone or IRFs and STAT2 were overexpressed. Renilla luciferase was used as an internal control. (F) The indicated protein and mRNA expression levels were determined in sgControl, sgSTAT2 and sgIRF9 HCT-116 cells. (G) The indicated protein levels were determined by immunoblotting when IRF1 was knocked down in sgNIPBL HCT-116 cells. (H) The indicated protein levels were determined by immunoblotting when STAT1 was knocked down in sgNIPBL HCT-116 cells. (I) Correlation of STAT2 and IRF9 mRNA levels with PD-L1 mRNA levels in TCGA colorectal tumour samples (n=457). Spearman's correlation and estimated significance were calculated with TIMER and adjusted for tumour purity. (J-K) The expression levels of the indicated protein and mRNA were determined by immunoblotting when STAT2 and IRF9 were co-expressed in HCT-116 cells. (L) The expression levels of the indicated protein and mRNA were determined by immunoblotting when STAT2 and IRF9 were co-expressed in sgSTAT1 or sgIRF1 293T cells. (M) STAT2 physically associated with IRF9. IgG was used as a negative control. (N) IRF9 selectively bound with STAT2 but not STAT1. (O) Dual immunofluorescent staining of STAT2 and IRF9 in

959 sgNIPBL HCT-116 cells. Scale bar, 20 μ m. (P) STAT2 and IRF9 co-occupied the
 960 PD-L1 promoter close to the -233-bp to -133-bp region in sgNIPBL HCT-116 cells
 961 according to a ChIP-PCR assay. (Q) The luciferase activity of the 1-kb PD-L1
 962 promoter with or without the 60-bp STAT2-IRF9 binding site was detected when
 963 STAT2 and IRF9 were individually expressed or co-expressed. (R) The luciferase
 964 activity of the 60-bp PD-L1 promoter was significantly elevated when STAT2 and
 965 IRF9 were co-expressed. (S) Indicated protein and mRNA expression were
 966 determined when IRF3 wild-type and 5D constitutive active mutant were exogenously
 967 expressed for 48 hours. (T) The luciferase activities of STAT2 and IRF9 promoters
 968 were determined when IRF3 wild-type and 5D mutant were exogenously expressed
 969 for 48 hours. (U) The mRNA expression levels of STAT2 and IRF9 were determined
 970 when IRF3 was knocked down. Data are shown as the mean $\pm$ SD, and the p value
 971 was calculated by two-tailed t tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <
 972 0.0001.
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