

NPC1 controls TGFBR1 stability in a cholesterol transport-independent manner and promotes hepatocellular carcinoma progression

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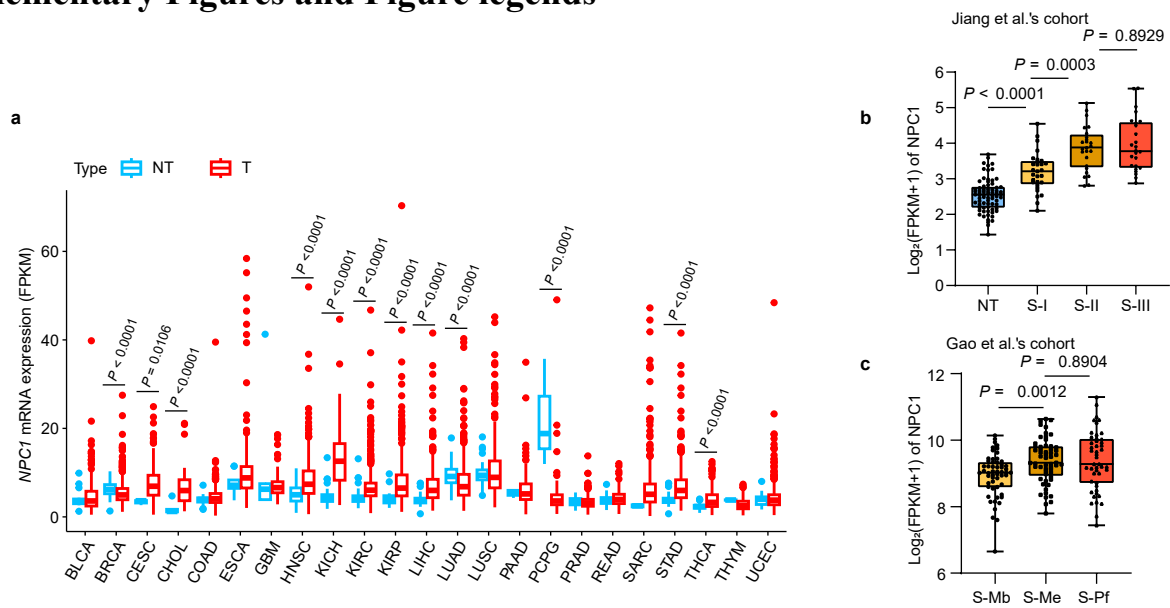
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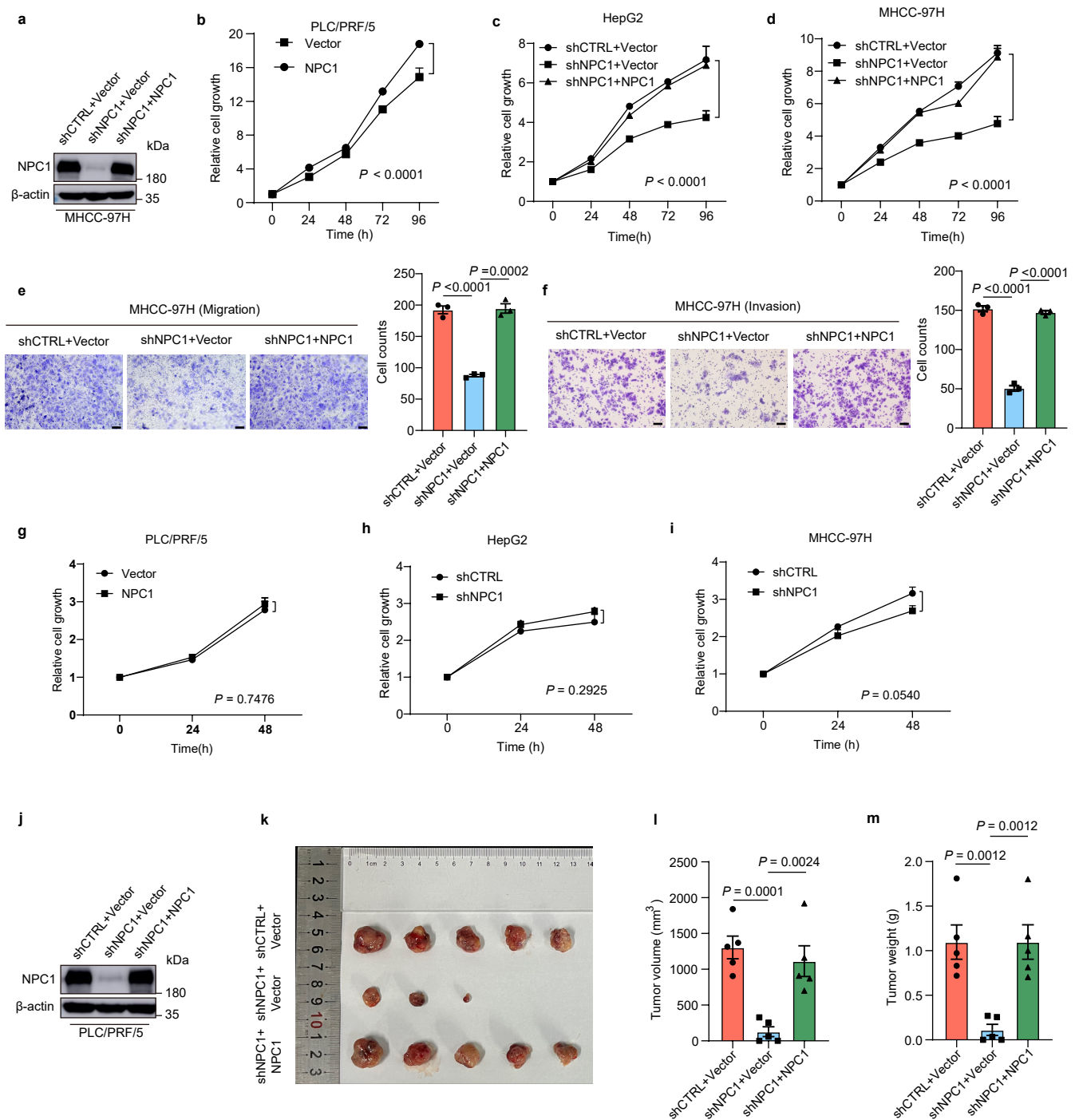
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Supplementary Figures and Figure legends

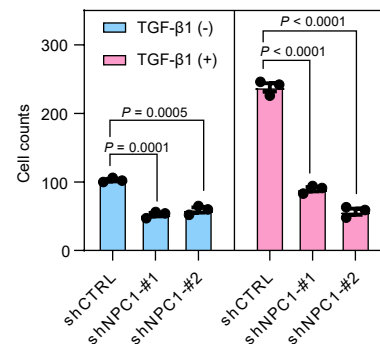
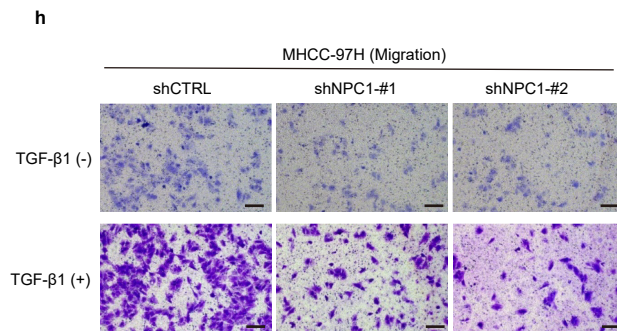
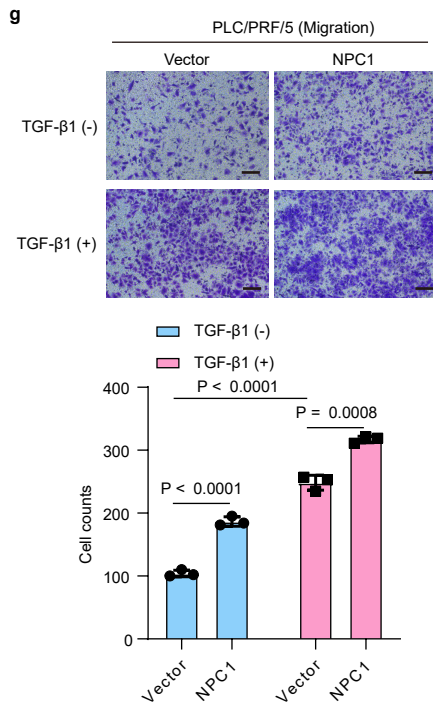
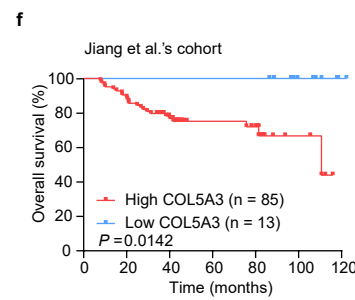
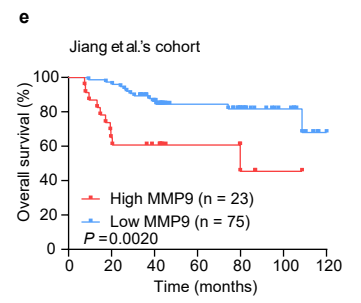
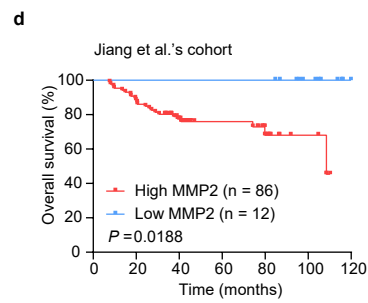
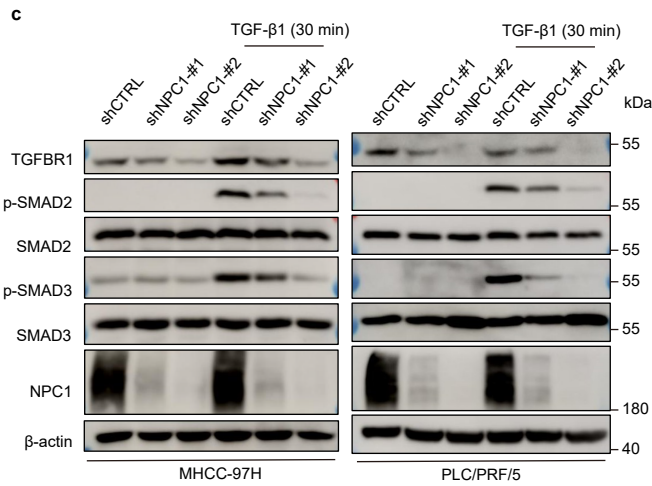
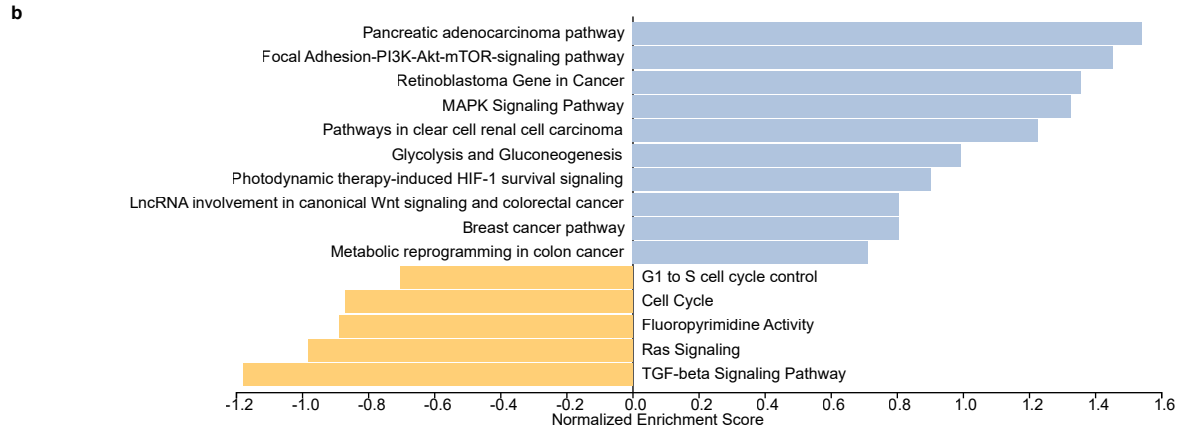
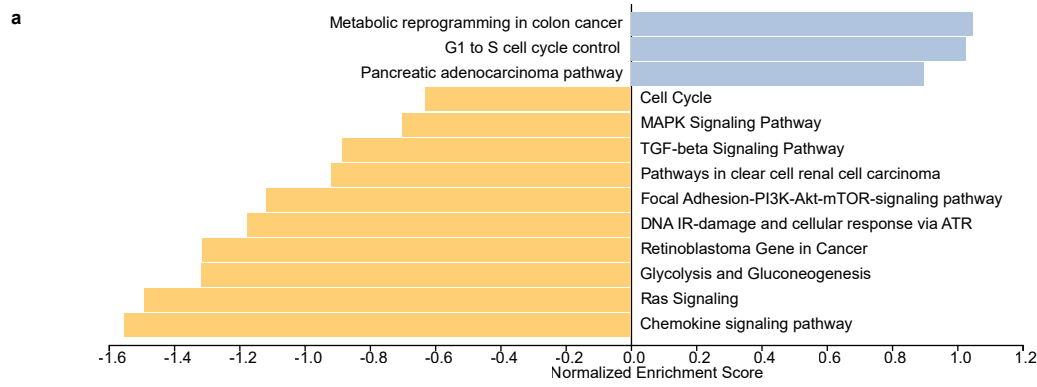


Supplementary Fig. 1 | NPC1 is upregulated in various cancers. a, *NPC1* mRNA expression between normal tissues adjacent to the tumor (NT) and tumor tissues (T) in 23 cancer types in TCGA datasets. The sample sizes are indicated in the source data. **b, c**, Upregulation of *NPC1* mRNA in paired non-tumor tissues (NT) and tumor tissues (T) in Jiang et al.'s cohort (**b**) (NT, n = 73; S-I, n = 29; S-II, n = 25; S-III n = 25) and Gao et al.'s cohort (**c**) (S-Mb, n = 55; S-Me, n = 57; S-Pf, n = 47). In the box plots, the middle bar represents the median, and the box represents the interquartile range; bars extend to 1.5× the interquartile range. Statistical significance was determined by Mann-Whitney U test (**a-c**). Source data are provided as a Source Data file.

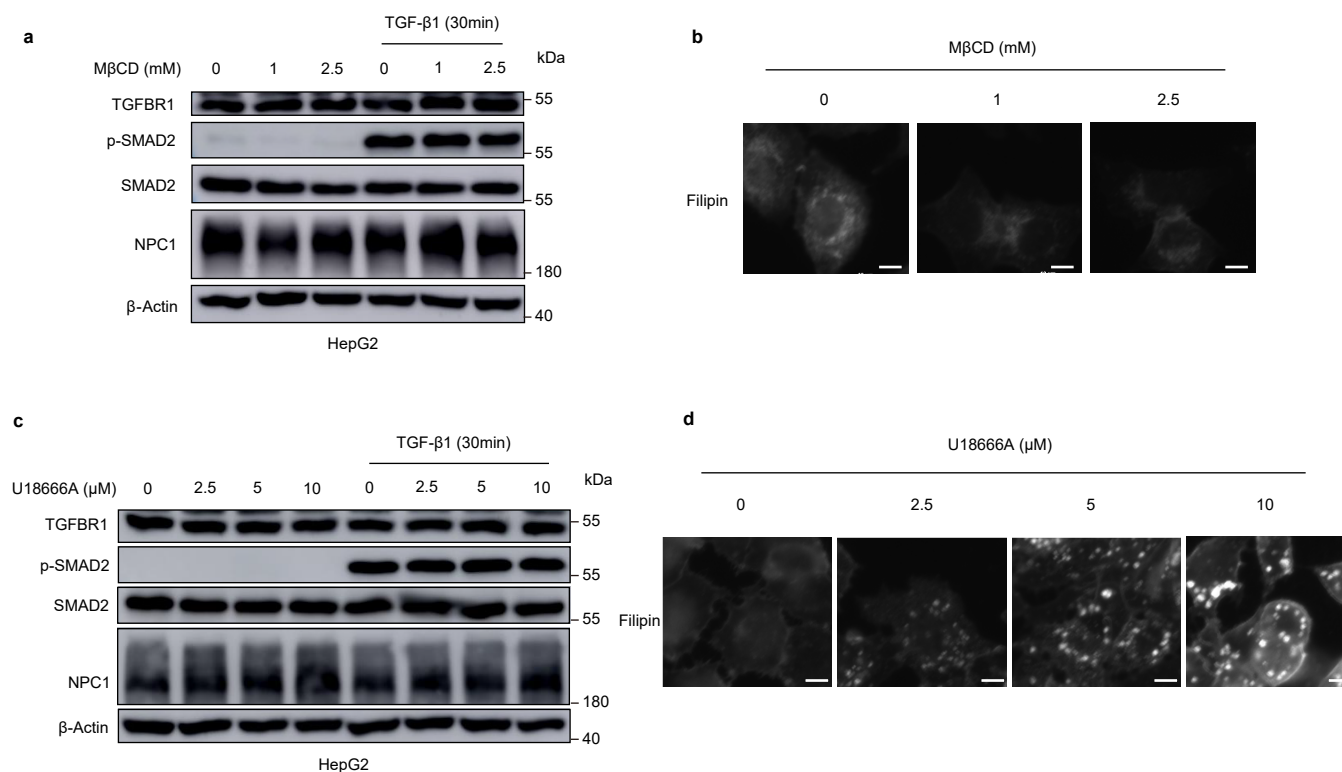


Supplementary Fig. 2 | NPC1 promotes HCC growth and metastasis. a, Confirmation of NPC1 knockdown and re-expression in HCC cells. **b-d,** Cell growth curves were measured in HCC cells with NPC1 stable overexpression (**b**) or knockdown (**c, d**). The relative cell number was expressed as fold change to Day 0. **e, f,** Transwell assay to examine the effect of NPC1 on HCC cell migration (**e**) or invasion (**f**); scale bars, 100 μ m. **g-i,** NPC1 didn't have significant effect on

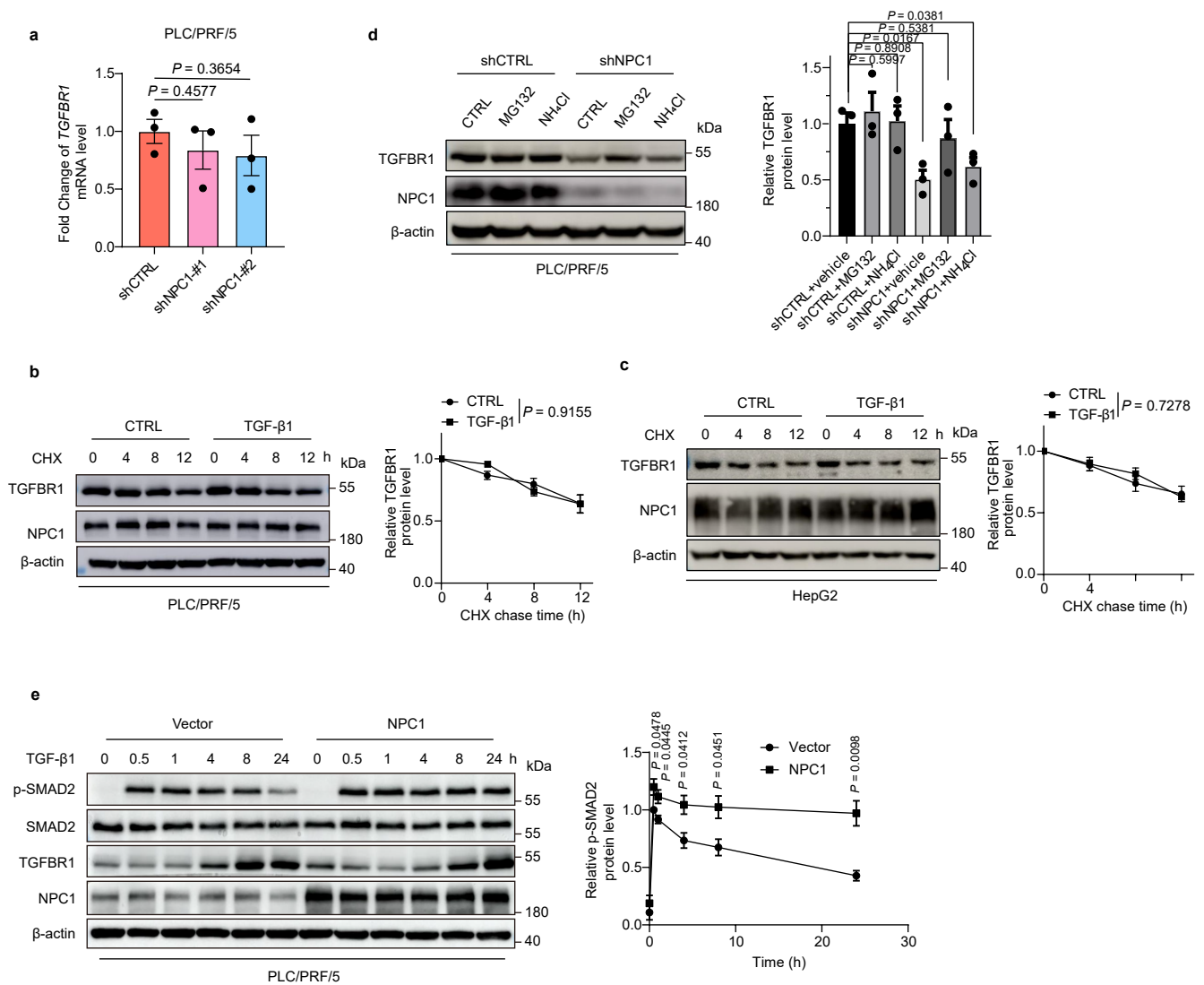
cell proliferation of HCC cells when they were cultured in serum free medium for 48 h. **j-m**, Confirmation of NPC1 knockdown and re-expression in PLC/PRF/5 cells (**j**). Photographs of xenograft tumors induced by the subcutaneous inoculation of NCG mice (n = 5 mice per group) with NPC1-knockdown PLC/PRF/5 cells with further overexpression of NPC1 (**k**). Graphs of xenograft tumor volumes (**l**), and xenograft tumor weights (**m**). Data are presented as the mean $\pm$ s.e.m. n = 3 (**b-i**) biologically independent samples. Statistical significance was determined by two-way analysis of variance (ANOVA) (**b-d**) or two-tailed unpaired Student's t-test (**e-i, l-m**). **a-j** Data were verified in three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 3 | NPC1 regulates TGF- β pathway. a, b, Gene Set Enrichment Analysis (GSEA) of different expression genes (DEGs). Pathway alterations in the PLC/PRF/5 (**a**) or HepG2 (**b**) cell lines after the NPC1 knockdown. Upregulated and downregulated pathways are indicated in blue (right) and yellow (left) bars. **c**, Immunoblot analysis of TGFBR1, p-SMAD2, p-SMAD3, SMAD2, SMAD3 and NPC1 expression in MHCC-97H and PLC/PRF/5 cells with NPC1 stable knockdown. **d-f**, Kaplan–Meier overall survival curves of individuals with high or low MMP2 (**d**), MMP9 (**e**) or COL5A3 (**f**) expression. **g**, Transwell assay was performed in NPC1-overexpression PLC/PRF/5 cells with or without TGF- β 1 (10 ng/mL) treatment; scale bars, 100 μ m. **h**, Transwell assay was performed in NPC1-knockdown MHCC-97H cells with or without TGF- β 1 (10 ng/mL) treatment; scale bars, 100 μ m. Data are presented as the mean $\pm$ s.e.m. $n = 3$ (**a, b, g, h**) biologically independent samples. Statistical significance was determined by two-tailed unpaired Student's t-test (**g, h**) or log-rank test (**d-f**). **a, b, g, h** Data were verified in three independent experiments. Source data are provided as a Source Data file.

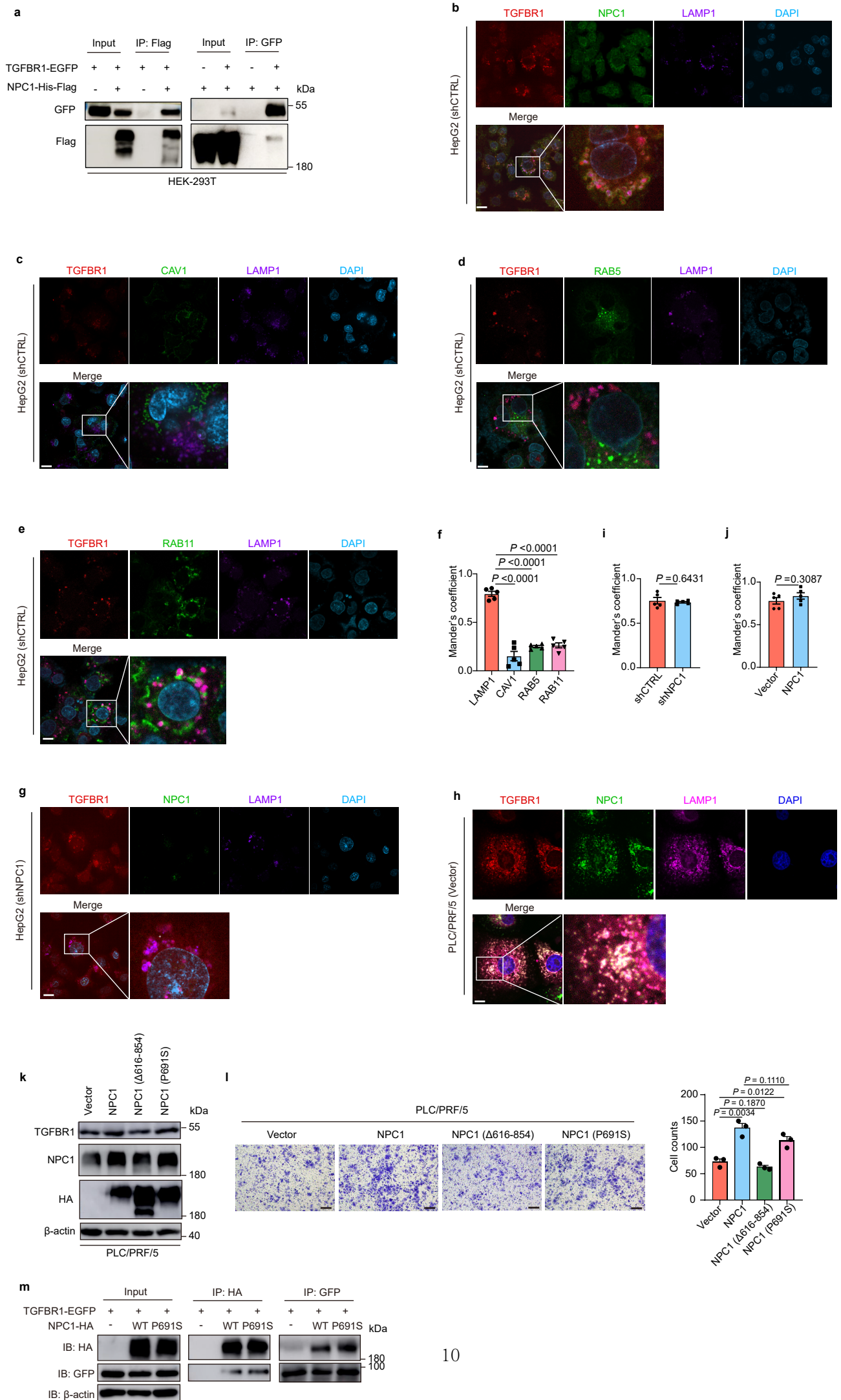


Supplementary Fig. 4 | NPC1 regulates TGF-β pathway in a cholesterol transport-independent manner. a-d, HepG2 cells were treated with MβCD (**a**) or U18666A (**c**), then analyzed by western blot to measure whole cell expression of TGFBR1 or the activity of TGF-β pathway. Cells related to (**a**, **c**) were fixed and stained with filipin to label free cholesterol accumulated in LE/Ly; scale bars, 10 μm (**b**, **d**). All experimental data were verified in three independent experiments. Source data are provided as a Source Data file.



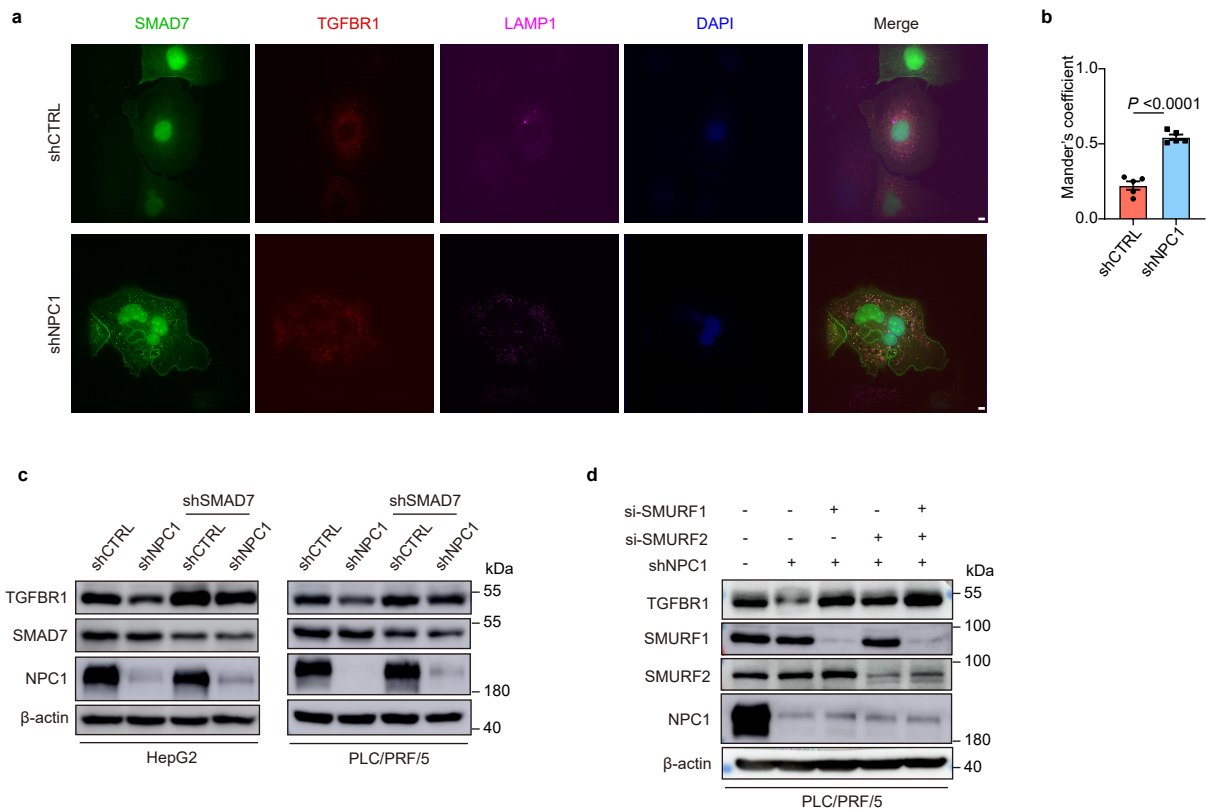
Supplementary Fig. 5 | NPC1 increases protein stability of TGFBR1 regardless of TGF- β 1 treatment. **a**, qPCR analysis of NPC1 and TGFBR1 mRNA levels in NPC1-knockdown PLC/PRF/5 cells. **b**, **c**, PLC/PRF/5 (**b**) or HepG2 (**c**) cells were treated with CHX with or without TGF- β 1 (10 ng/mL) for indicated times and then analyzed by western blot. **d**, PLC/PRF/5 cells with or without stable knockdown of NPC1 were treated with vehicle, MG132 (10 μ M), or NH₄Cl (10 mM) for 12 hours. Cell lysates were subjected to immunoblot with TGFBR1 or NPC1 antibody. **e**, PLC/PRF/5 cells with stable overexpression of NPC1 were stimulated with TGF- β 1 (10 ng/mL) for the times indicated. Levels of p-SMAD2, SMAD2, TGFBR1 and NPC1 were assayed by western blot. Data are presented as the mean $\pm$ s.e.m. $n = 3$ (**a-e**) biologically independent samples.

Statistical significance was determined by two-tailed unpaired Student's t-test (**a, d, e**) or two-way analysis of variance (ANOVA) (**b, c**). All experimental data were verified in three independent experiments. Source data are provided as a Source Data file.



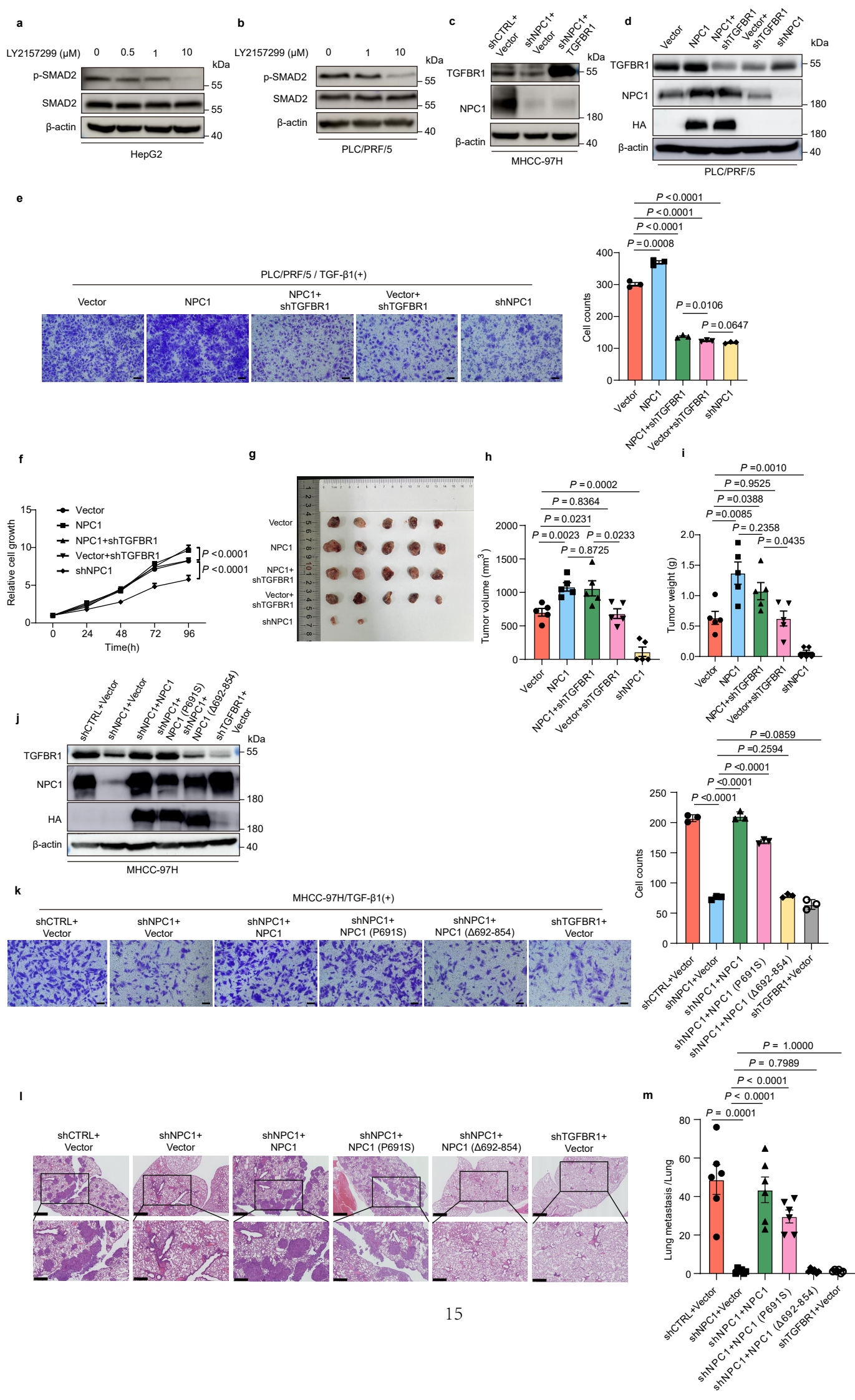
Supplementary Fig. 6 | NPC1 interacts with TGFBR1. **a**, The lysates of HEK-293T transfected with indicated constructs were subjected to immunoprecipitation with anti-Flag (or GFP) antibody. The immunoprecipitates were then immunoblotted with anti-GFP (or Flag) antibody. **b**, HepG2 cells stably expressing TGFBR1-mCherry-His were immunostained with antibodies against NPC1 and LAMP1 to determine the colocalization among TGFBR1, NPC1 and LAMP1 in HepG2 cells. **c**, HepG2 cells stably expressing TGFBR1-mCherry-His and Caveolin-1 (CAV1)-Myc were immunostained with antibodies against Myc and LAMP1 to determine the colocalization among TGFBR1, CAV1 and LAMP1 in HepG2 cells. **d-e**, HepG2 cells stably expressing TGFBR1-mCherry-His were immunostained with antibodies against RAB5 (**d**) /RAB11 (**e**) and LAMP1 to determine the colocalization among TGFBR1, RAB5/ RAB11 and LAMP1 in HepG2 cells. **f**, Quantification of the colocalization between these four proteins (LAMP1, CAV1, RAB5 and RAB11) and TGFBR1 using the Mander's coefficient. **g**, NPC1-knockdown HepG2 cells stably expressing TGFBR1-mCherry-His were immunostained with antibodies against NPC1 and LAMP1 to determine the colocalization among TGFBR1, NPC1 and LAMP1 in HepG2 cells. **h**, PLC/PRF/5 cells stably overexpressing TGFBR1-mCherry-His and HA-tag were immunostained with antibodies against NPC1 and LAMP1 to determine the colocalization among TGFBR1, NPC1 and LAMP1 in PLC/PRF/5 cells. Representative images from three independent experiments (**b-e**, **g-h**) are shown; scale bars, 10 μ m. **i-j**, Quantification of the colocalization between TGFBR1 and

LAMP1 using the Mander's coefficient in HepG2 cells with NPC1 stable knockdown (**i**) or PLC/PRF/5 cells with NPC1 stable overexpression (**j**). **k**, Immunoblot analysis of TGFBR1 and NPC1 expression in PLC/PRF/5 cells with overexpression of NPC1, NPC1 (Δ 616-854), or NPC1 (P691S). **l**, Transwell assay was performed in cells related to (**k**) (n = 3 biologically independent samples); scale bars, 100 μ m. **m**, NPC1-HA or NPC1 (P691S)-HA stable overexpression PLC/PRF/5 cells transfected with TGFBR1-EGFP plasmid were subjected to immunoprecipitation with anti-HA or anti-GFP magnetic beads. The lysates and immunoprecipitates were then blotted. Data are presented as the mean $\pm$ s.e.m. Statistical significance was determined by two-tailed unpaired Student's t-test (**f**, **i**, **j**, **l**). All experimental data were verified in three independent experiments. Source data are provided as a Source Data file.

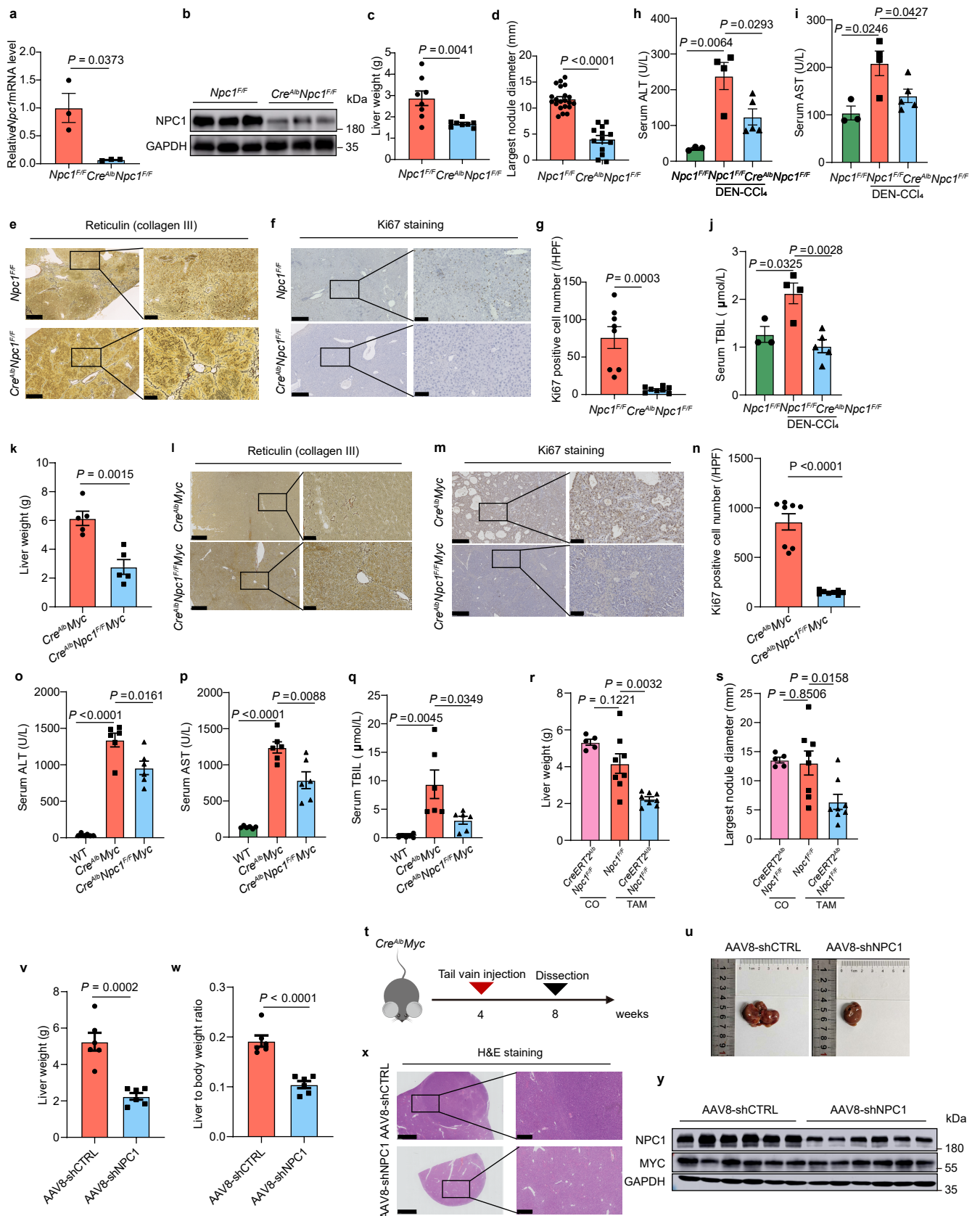


Supplementary Fig. 7 | NPC1 regulates TGFBR1 in a SMAD7-dependent manner. **a**, PLC/PRF/5 cells stably expressing TGFBR1-mCherry-His and SMAD7-EGFP were immunostained with antibodies against LAMP1 and then imaged by Structure Illumination Microscopy to determine the colocalization among TGFBR1, SMAD7 and LAMP1 in PLC/PRF/5 cells with NPC1 stable knockdown. The images were collected through the Polar-SIM system. **b**, Quantification of the colocalization between TGFBR1 and SMAD7 using the Mander's coefficient in PLC/PRF/5 cells with NPC1 stable knockdown. **c**, Immunoblot analysis of TGFBR1, SMAD7 and NPC1 expression in NPC1-knockdown HepG2 and PLC/PRF/5 cells with or without knockdown of SMAD7. **d**, Immunoblot analysis of TGFBR1, SMURF1, SMURF2 and NPC1 expression in NPC1-knockdown PLC/PRF/5 cells were transfected with specific siRNAs

targeting SMURF1 or SMURF2. Representative images from three independent experiments **(a)** are shown; scale bars, 10 μm . Data are presented as the mean $\pm$ s.e.m. Statistical significance was determined by two-tailed unpaired Student's t-test **(b)**. All experimental data were verified in three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 8 | TGFBR1 is crucial for NPC1-mediated promotion of HCC progression. **a, b**, HepG2 (**a**) and PLC/PRF/5 (**b**) cells with or without LY2157299 treatment for 16 h were pretreated with TGF- β 1 (10 ng/mL) for 30 min before collection. Cell lysates were subjected to immunoblot with p-SMAD2 or SMAD2 antibody. **c**, Immunoblot analysis of TGFBR1 and NPC1 expression in NPC1-knockdown MHCC-97H cells with or without TGFBR1 overexpression **d**, Immunoblot analysis of TGFBR1 and NPC1 expression in PLC/PRF/5 cells with stable overexpression or knockdown of indicated genes. **e**, Transwell assay was performed in cells related to (**d**); scale bars, 100 μ m. **f**, Cell growth curves were measured in indicated cells. **g-i**, Photographs of xenograft tumors induced by the subcutaneous inoculation of NCG mice (n = 5 mice per group) in indicated groups (**g**). Graphs of xenograft tumor volumes (**h**), and xenograft tumor weights (**i**). **j**, Immunoblot analysis of TGFBR1 and NPC1 expression in MHCC-97H cells with stable overexpression or knockdown of indicated genes. **k**, Transwell assay was performed in cells related to (**j**); scale bars, 100 μ m. **l-m**, 1×10^6 Luciferase-expressing MHCC-97H cells were injected into NCG mice by tail vein. The mice were euthanized 8 weeks later by a cervical dislocation. Representative H&E staining images of lung tissues are shown; scale bars, 1mm; insets: twofold magnification; scale bars, 500 μ m (**l**). The incidence of lung metastasis in mice (**m**) (n = 6 mice per group). Data are presented as the mean $\pm$ s.e.m. n = 3 (**e, f, k**) biologically independent samples. Statistical significance was determined by two-tailed unpaired Student's t-test (**e, h, i, k, m**) or two-way



Supplementary Fig. 9 | Hepatic NPC1 deficiency inhibits HCC tumorigenesis

a, *Npc1* mRNA expression in *Npc1^{F/F}* and *Cre^{Alb}Npc1^{F/F}* mouse HCC tissues (n = 3 mice per group). **b**, NPC1 protein expression in *Npc1^{F/F}* and *Cre^{Alb}Npc1^{F/F}* mouse HCC tissues (n = 3 mice per group). **c, d**, liver weight (n = 8 mice) (**c**), diameters of the largest nodules (n = 21 mice in *Npc1^{F/F}* and n = 13 mice in *Cre^{Alb}Npc1^{F/F}* groups) (**d**) of the indicated mice. **e**, Representative reticulin staining images of the indicated mouse livers. Scale bars: left panels, 500 μ m; right panels, 100 μ m. **f**, Representative IHC staining images of Ki67 in the indicated mouse livers. Scale bars: left panels, 500 μ m; right panels, 100 μ m. **g**, Quantification of Ki67 staining in the indicated mouse livers (n = 8 mice). **h-j**, Analysis of serum ALT (**h**), AST (**i**) and TBIL (**j**) levels in normal *Npc1^{F/F}* (n = 3), *Npc1^{F/F}* (n = 4) and *Cre^{Alb}Npc1^{F/F}* (n = 5) mice with DEN-CCl₄ induced HCC. **k**, liver weight of *Cre^{Alb}Myc* and *Cre^{Alb}Npc1^{F/F}Myc* mice (n = 5 mice). **l**, Representative reticulin staining images of the indicated mouse livers. Scale bars: left panels, 500 μ m; right panels, 100 μ m. **m**, Representative IHC staining images of Ki67 in the indicated mouse livers. Scale bars: left panels, 500 μ m; right panels 100 μ m. **n**, Quantification of Ki67 staining in the indicated mouse livers (n = 8 mice). **o-q**, Analysis of serum ALT (**o**), AST (**p**) and TBIL (**q**) levels in WT, *Cre^{Alb}Myc* and *Cre^{Alb}Npc1^{F/F}Myc* mice (n = 6 mice). **r, s**, liver weight (**r**), diameters of the largest nodules(**s**) of *Npc1^{F/F}* mice treated with coil (CO) (n = 5 mice), *CreERT2^{Alb}Npc1^{F/F}* mice treated with coil (CO) or tamoxifen (TAM) (n = 8 mice). **t**, Schematic view of the treatment plan using AAV serotype 8 (AAV8).