Supplementary Information

Epigenetic restriction of Hippo signaling by MORC2 underlies stemness of hepatocellular carcinoma cells

Tao Wang[1, #], Zhong-yi Qin[1, #], Liang-zhi Wen[1, #], Yan Guo[1], Qin Liu[1], Zeng-jie Lei[2], Wei Pan[3], Kai-jun Liu[1], Xing-wei Wang[1], Shu-jie Lai[1], Wen-jing Sun[1], Yan-ling Wei[1], Lei Liu[1], Ling Guo[1], Yu-qin Chen[1], Jun Wang[1], Hua-liang Xiao[4], Xiu-wu Bian[5], Dong-feng Chen[1, *] and Bin Wang[1,*]

Author affiliations: [1]Department of Gastroenterology, [4]Department of Pathology, Institute of Surgery Research, Daping Hospital, Third Military Medical University (Army Medical University), Chongqing 400042; [2]Department of Medical Oncology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, 210002; [3]Department of Medical Genetics, Second Military Medical University (Navy Medical University), Shanghai 200433; [5]Institute of Pathology and Southwest Cancer Center, Key Laboratory of Tumor Immunopathology of Ministry of Education of China, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038; P. R. China;

# These authors contributed equally to the study.

Supplementary Material and Methods

Cell culture

Human embryonic kidney cell line (HEK-293), immortalized hepatocyte L02 cell line, and HCC cell lines (HepG2, Huh7, 7721, PLC/PRF/5 (PLC)) were purchased from the Shanghai Cell Collection (Shanghai, China). All cell lines were maintained in DMEM media (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo scientific, USA), 100 units/ml penicillin, 100 μg/ml streptomycin and 4mM glutamine, in a 5% CO_2 and humidified atmosphere at 37°C. If there are no other special instructions, cells were harvested at approximate subconfluence. 5-Aza-2'-deoxycytidine (5-Aza) was purchased from Sigma-Aldrich (Germany), dissolved in DMSO and diluted to indicated concentration in cell culture media.

Co-immunoprecipitation (Co-IP) assay

For Co-IP analysis, 1mg of lysates were incubated with indicated antibodies (Supplementary Table 7) for 4 hrs at 4°C, then incubated with Protein A/G sepharose beads (GE Healthcare). After washing five times with NETN buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% NP-40), the immunoprecipitation product was subjected to western blot assay.

Patients and HCC tissues

Written informed consent was obtained from individual patients whose tissue and clinical data were involved. The experimental protocols were approved by the Institutional Review Board of Daping Hospital of Third Military Medical University (Chongqing, China). HCC and adjacent non-tumor live tissues were collected from 200 patients who were diagnosed as primary HCC and underwent curative hepatectomy at department of Hepatology of Daping Hospital between 2005 and 2008. The diagnosis of HCC was based on pathological inspection and TNM stage criteria were according to UICC/AJCC, 2010. Patients with secondary liver cancer or those received radiotherapy or chemotherapy prior to surgery were not included. The demographic and clinical characteristics of patients are summarized in Supplementary Table 3. Tissue specimens were fixed by formalin and paraffin-embedded for IHC or HE examinations. Some fresh specimens were collected for qRT-PCR (Supplementary Table 1), western blot (Supplementary Table 2) or MeDIP-qPCR assay (Supplementary Table 4).

Gene silencing and ectopic expression

To knockdown endogenous NF2 or KIBRA expression, human *NF2* or *KIBRA* specific shRNA oligonucleotides were synthesized and cloned into pLKO.1 puro vector (Addgene plasmid ID 8453) (*KIBRA* shRNA, 5'-CCGGTCAGATTGCGCCTTCGATATGCTCGAGCATATCGAAGGCGCA ATCTGATTTTTG-3'; *NF2* shRNA, 5'-CCGGGCTCTGGATATTCTGCACAATCTCGAGATT GTGC AGAATATCCAGAGCTTTTTG-3'; *YAP1* shRNA, 5'-CCGGGACCAATAGCTCAGAT CCTTTCTCGAGAAAGGATCTGAGCTATTGGTCTTTTTG-3'; *TAZ* shRNA, 5'-CCGGCGGAC TTCATTCAAGAGGAATCTCGAGATTCCTCTTGAATGAAGTCCGTTTTTG-3'; *TP53* shRNA, 5'-CCGGCGGCGCACAGAGGAAGAGAATCTCGAGATTCTCTTCCTCTGTGCGCCGTTTTT-3'; *SMAD4* shRNA, 5'-CCGGGTACTTCATACCATGCCGATTCTCGAGAATCGGCATGGTA TGAAGTACTTTTTG-3'). Lentivirus packaging, cell transduction and puromycin selection was performed as previously described.[1] Cells infected with lentivirus delivering a scramble shRNA

(shScramble) served as a negative control. To deplete several DNA methylation regulatory enzymes, siRNA duplexes targeting *DNMT1* (ID #110914), *DNMT3A* (ID #220178), *DNMT3B* (ID #111746), *TET1* (ID #147892), *TET2* (ID #126964), *TET3* (ID #264556) as well as a non-targeting siRNA control (Ambion, UK) were transfected at a concentration of 20 nM, using Lipofectamine 2000 (Invitrogen, UK), according to the manufacturer's instructions.

For ectopic gene expression, cDNA clone of *MORC2* (SC114779), *NF2* (SC124024), *KIBRA* (SC107042), *DNMT3A* (SC307007), was purchased from Origene, USA. The ORF was amplified by PCR and digested by BglII and XhoI. Then the ORF fragment was ligated into BamHI and XhoI digested lentiviral vector pLentis-Bid-PGK-PURO-CTE-TKpA-SFFV-MCS (Lentis Bioscience, China) to generate respective expressing vector, in which the expression of target genes were driven by SFFV promoter. The plasmids were verified using DNA sequencing carried out by Life Technologies, USA. Subsequently, 20 μg of the plasmids were cotransfected with 5 μg pMD2.G, 15 μg psPAX2 into 293FT cells in 10-cm cell dish to generate lentiviral particles. Cells were transduced with lentivirus at a MOI of 20, and successfully transduced cells were selected using puromycin. The Flag-tagged *MORC2* mutant (D68A) was generated using Phusion Site-Directed Mutagenesis Kit (Thermo Scientific, USA), according to the manufacturer's instructions.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA of cultured cell lines or tissue was extracted by TRIzol reagent (Invitrogen, USA), and subsequently reverse transcribed to cDNA using primeScript^{TM} RT kit (Takara, Japan). Triplicate qPCR was performed using a SYBR premix Ex Taq^{TM} Green II (Takara, Japan) to detect the relative mRNA level of indicated genes. Primers sets were listed in Supplementary Table 7. β-actin was used as internal control to calculate fold change of indicated genes expression level. The assays were performed in triplicate.

Immunohistochemical (IHC) staining and scoring system

Samples from 200 (MORC2 staining) or 100 (YAP, NF2, KIBRA and stem markers staining) patients were involved in IHC assay. The paraffin-embedded tissue sections (4 μm) were subjected to standard procedure to remove paraffin and antigen retrieval in sodium citrate 10 mmol/L (pH 6.0 for NF2, KIBRA, YAP, Ki67, CD133 and MORC2) or EDTA (pH 8.0 for Lgr5 and Nanog). After blocking in goat serum for 30 min at 37°C, tissue sections were incubated overnight at 4°C with indicated primary antibodies (Supplementary Table 6). HRP-conjugated secondary antibody was incubated for 1h, 37°C. The signal was visualized by DAB (DAKO, China). Images were acquired by Olympus BX50 microscope and 10 representative fields were used to evaluate the expression of indicated proteins using the following scoring system. Staining strength was scored as 0 (negative), 1 (weak), 2 (moderate), 3 (strong), and distribution was scored as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (76%-100%) by positive staining area. Final score of each sample was obtained by multiplying the strength score by the distribution score, which was evaluated by two double-blinded pathologists separately. Subsequently, MORC2^{low} (IHC scoring < 6) and MORC2^{high} (IHC scoring ≥ 6) was defined and used in survival analysis of HCC patients.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100/PBS solution for 15min at room temperature. After blocking with normal goat serum for 1h

at 37°C, cells were incubated with primary antibodies (Supplementary Table 6) overnight at 4°C, followed by Alexa^{TM}594-conjugated secondary antibody (Life technologies, USA) incubation and DAPI (Invitrogen, USA) counterstaining. Immunofluorescence images were acquired by laser confocal scanning microscope (ZEISS, Germany).

Western blot analysis

Cells were harvested and total protein was extracted by RIPA Lysis and Extraction Buffer (Thermo Scientific USA). Protein concentration was determined by RC-DC™ Protein Assay Kit (Bio-Rad, USA). A total of 50 μg protein in each sample was separated by SDS-PAGE, then electrotransferred to PVDF membrane (Millipore, USA). After blocking in TBST with 5% BSA, the membrane was incubated overnight with primary antibody (Supplementary Table 6) at 4°C and HRP-labeled secondary antibody at 37°C, for 2 hr. β-actin was used as a loading control. The signal was visualized with Immobilon Western Chemiluminescent HRP Substrate detection reagents (Millipore, USA). Images were acquired on a ChemiDoc™ imaging System (Bio-Rad, USA).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using EZ-ChIP^{TM} chromatin immunoprecipitation kit, according to manufacturer's instructions. Briefly, $3x10^6$ cells were treated with 1% formaldehyde to crosslink the proteins to the DNA. Then cell lysis was subjected to sonication to acquire 200-1000 bp length DNA fragments for subsequent immunoprecipitation with the primary antibodies listed in Supplementary Table 7. After DNA purification, ChIP product was used in PCR assay, using genomic DNA as positive input. Primers for ChIP analysis are listed in Supplementary Table 7. All experiments were performed in triplicate.

Luciferase reporter assay

The activity of YAP in individual cell lines was examined by 8xGTIIC-luciferase reporter assay.[2] This YAP/TEAD-responsive reporter plasmid was obtained from Addgene (Plasmid #34615). Cells (4×10^4 cells/well) were cultured in 24-well tissue culture plates and cotransfected with 8xGTIIC-luciferase and *Renilla* luciferase reporters at subconfluent density. Cells were harvested after 24 hr with reporter lysis buffer, and the signals were normalized to the *Renilla* luciferase signal. The experiments were performed in triplicate.

Methylation analysis

For methylation-specific PCR (MSP), cell DNA was extracted and bisulphite modified as previously reported.[3] After purification with Wizard^{TM} DNA purification resin (Promega, USA), the bisulphite modified DNA was subjected to PCR amplification[3-7] using primers listed in Supplementary Table 7. Amplification was carried out for 35 cycles (45 sec at 95°C, 45 sec at the primer-specific annealing temperature, and 30 sec at 72°C), followed by a final 4 min extension at 72°C.[3] Bisulfite sequencing (BSP) was performed to quantify the population of methylated alleles, bisulphite-modified DNA was PCR-amplified using a set of degenerate primer pairs that amplify both methylated and unmethylated alleles.[3, 5] PCR-amplified fragments were subcloned into TA cloning vector and alteration of DNA was analyzed by DNA sequencing.

Alternatively, methylated DNA immunoprecipitation (MeDIP) analysis was performed with SimpleDIP™ Methylated DNA IP (MeDIP) Kit (#76853, CST, USA), according to the

manufacturer's instructions. Briefly, tissue or cell DNA was extracted using phenol-chloroform. After shearing and quantification, the extracted genomic DNA was immunoprecipitated with anti-5mC antibody, or IgG control antibody and ChIP-Grade protein G magnetic beads. The product was washed for 3 times and placed in a thermomixer for 30 min at 65°C with gentle vortexing to elute DNA from the antibody/protein G beads. After DNA purification procedure, qPCR was performed using the primers listed in Supplementary Table S7. Diluted 10% input genomic DNA was used as control to determine the efficiency of amplification.

Soft agar colony formation assay

To examine the tumorigenicity of HCC cells *in vitro*, 2 ml/well of 1% agar containing medium was pre-coated in 6-well plate, covered by 2ml 0.5% agar containing medium with 3×10^3 cells suspended. Cells were incubated for 2 to 4 weeks at 37°C. The colonies were stained by 1 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich, Germany) and those with a diameter greater than 75 μm were counted. The experiments were repeated for three times.

Sorafenib resistance assay

To determine sorafenib resistance of HCC cell lines, 4×10^2 cells/well PLC cells or 6×10^2 cells/well HepG2 cells were seeded in 6-well plate. After adherence, cells were treated with sorafenib (4 μM for HepG2 cells and 8 μM for PLC cells) for 48 hrs and cultured in normal medium for another 10 days to examine colony formation capacity. Colonies were fixed in 4% formaldehyde and subsequently stained by crystal violet (Solarbio, China). Experiments were performed in triplicate.

Flow cytometry

Flow cytometry analysis of HCC cells were performed as described previously.[1, 8] Briefly, cells were collected and washed with PBS 3 times, followed by incubation for 30 min at 37°C with Lgr5-PE or isotype control antibodies. Cells were stained with 7-amino-actinomycin D (7-AAD, eBioscience). The percentages of $Lgr5^+$ cells were analyzed in a BDAria II Sorter (BD Biosciences) by gating on $7\text{-}AAD^-$ living cells. Experiments were performed in triplicate.

Tumorsphere formation assay

For tumorsphere culture, indicated numbers of cells were cultured in ultra-low attached 24-well plates (Corning, USA). Cells were maintained in stem cell medium (DMEM/F12 containing 20 ng/mL EGF (PeproTech, USA), 20 ng/mL bFGF (PeproTech, USA), 10 ng/mL B27 (GIBCO, USA), 10 ng/mL HGF (Peprotech, USA), 4 μg/mL insulin (Sigma-Aldrich, Germany), and 1% methyl cellulose (Sigma-Aldrich, Germany)) for 2 weeks, fresh medium was added every 4 days.[1] At least 20 tumorspheres for each group were randomly selected to calculate the average diameters of tumorspheres.

Mouse xenograft tumor model

The animal experimental protocols were approved by the Animal Studies Ethics Committee of Third Military Medical University, Chongqing, China. SPF grade 4-week-old female nude mice were housed in individually ventilated cages. Approximately, 1×10^6 cell suspension were mixed with Matrigel™ (1:1) and then inoculated subcutaneously into the flanks of the mice. Length (*l*) and width (*w*) of subcutaneous tumors were measured weekly using a caliper and the volume was

calculated as $V=lw^2/2$. Four to six weeks later, mice were sacrificed, and the xenografts were excised, weighed, and imaged or fixed with 10% buffered formalin for further analysis.

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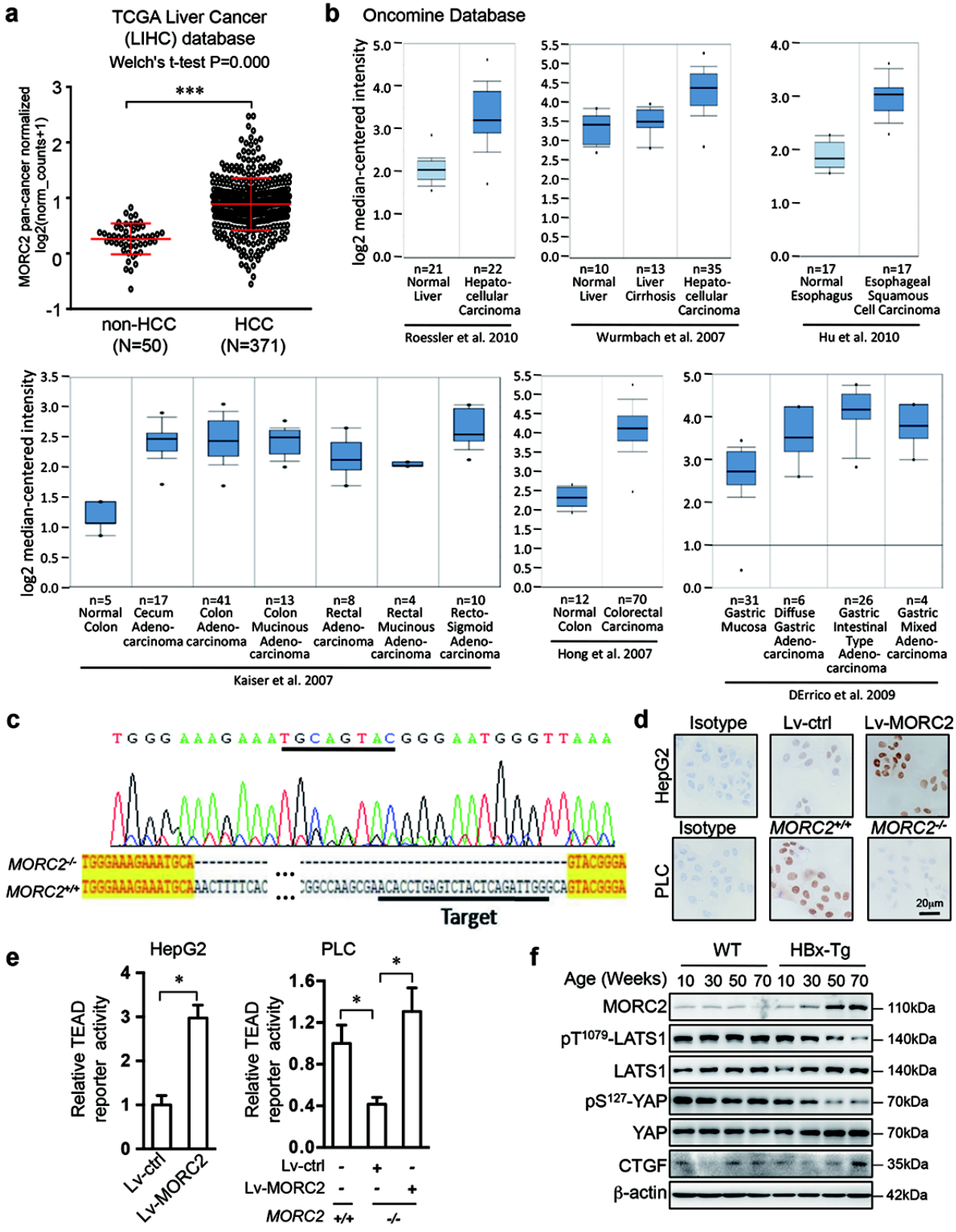
Supplementary Figures



Supplementary Figure 1. See next page for caption

Supplementary Figure 1. MORC2 inhibits Hippo signaling during liver tumorigenesis.

(**a, b)** MORC2 mRNA levels were elevated in multiple gastrointestinal tumors including HCC. Quantitative analysis of MORC2 mRNA levels in the RNA sequencing results from several independent HCC and other gastrointestinal cancer cohorts in the TCGA and Oncomine databases.

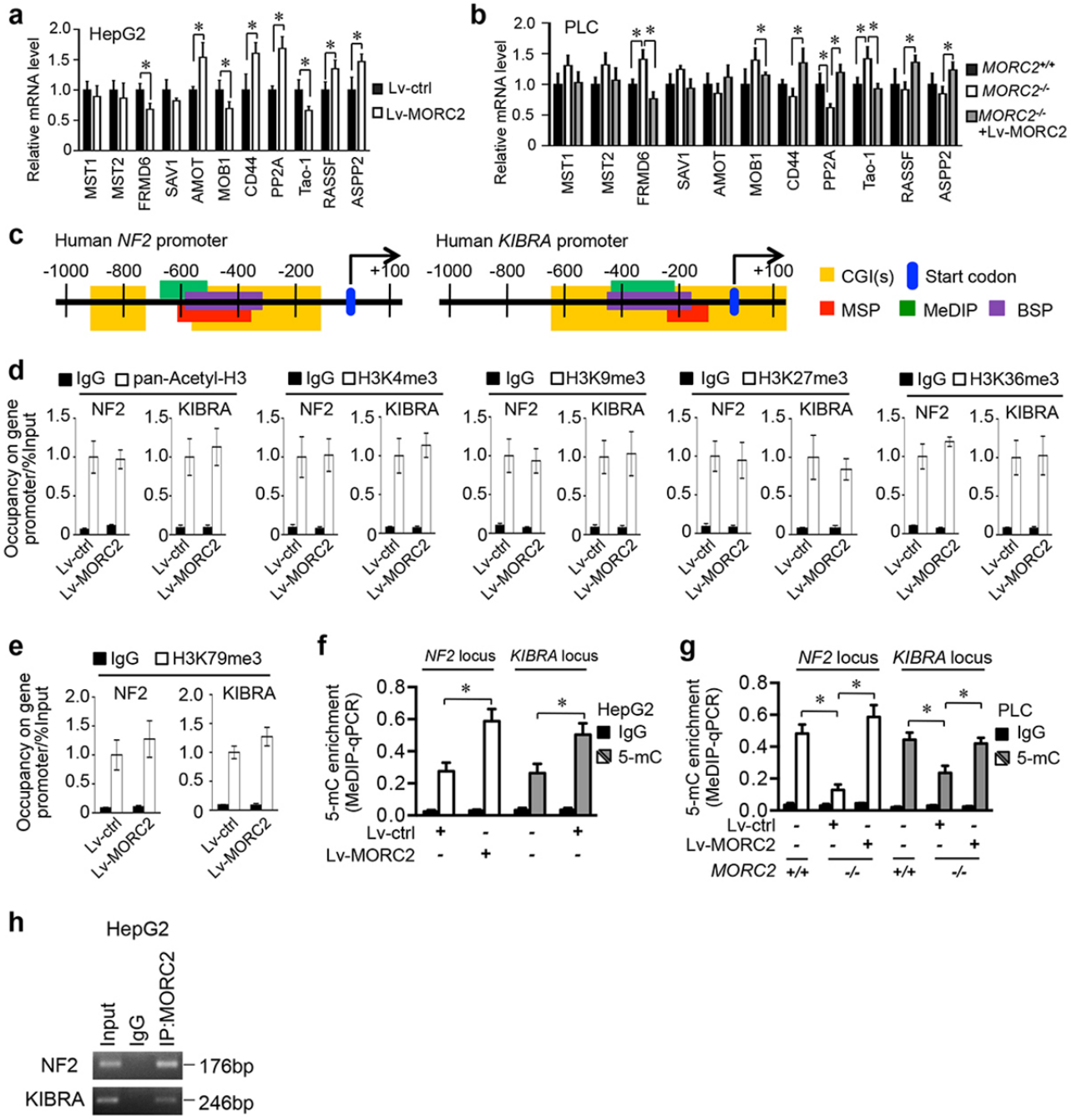
(c) A schematic depicting the CRISPR/Cas9 strategy applied to generate MORC2 knockout cell line. sgRNA sequence is underlined.

(d) The MORC2 antibody specifically recognized ectopically and endogenously expressed MORC2 protein in cells. Representative IHC images showing various levels of anti-MORC2 staining in control or HepG2 cells infected MORC2-expressing lentivirus, or $MORC2^{+/+}$ or $MORC2^{-/-}$ PLC cells. The rabbit isotype antibody served as a negative control.

(e) The levels of luciferase activity of 8x-GTIIC (TEAD) reporter were detected in HepG2 or 7404 cells infected with indicated lentivirus.

(f) Western blot analysis of MORC2 expression and Hippo signaling activity in the liver tissues derived from wild type or *HBx* transgenic (*HBx*-Tg) C57BL/6 mice at indicated age.

Representative images are presented in **d**, **f** and data are shown as the mean ± SD from three separate experiments (**e**). *, $P<0.05$.



Supplementary Figure 2. MORC2 promotes DNA methylation of *NF2* and *KIBRA* loci, with minimal effects on other epigenetic modifications.

(**a, b**) MORC2 minimally affects the mRNA levels of other Hippo pathway components. qRT-PCR analysis of Hippo signaling components using HepG2 cells that were infected with control or MORC2-expressing lentivirus, or various PLC cells (*MORC2*$^{+/+}$ or *MORC2*$^{-/-}$ or *MORC2*$^{-/-}$ cells expressing ectopic MORC2).

(**c**) Schematic diagrams showing relative genomic localization of primer sets used for amplifying the *NF2* and *KIBRA* loci in the methylation-specific PCR (MSP), methylated DNA immunoprecipitation and qPCR (MeDIP-qPCR) and Bisulfite sequencing PCR (BSP) assays.

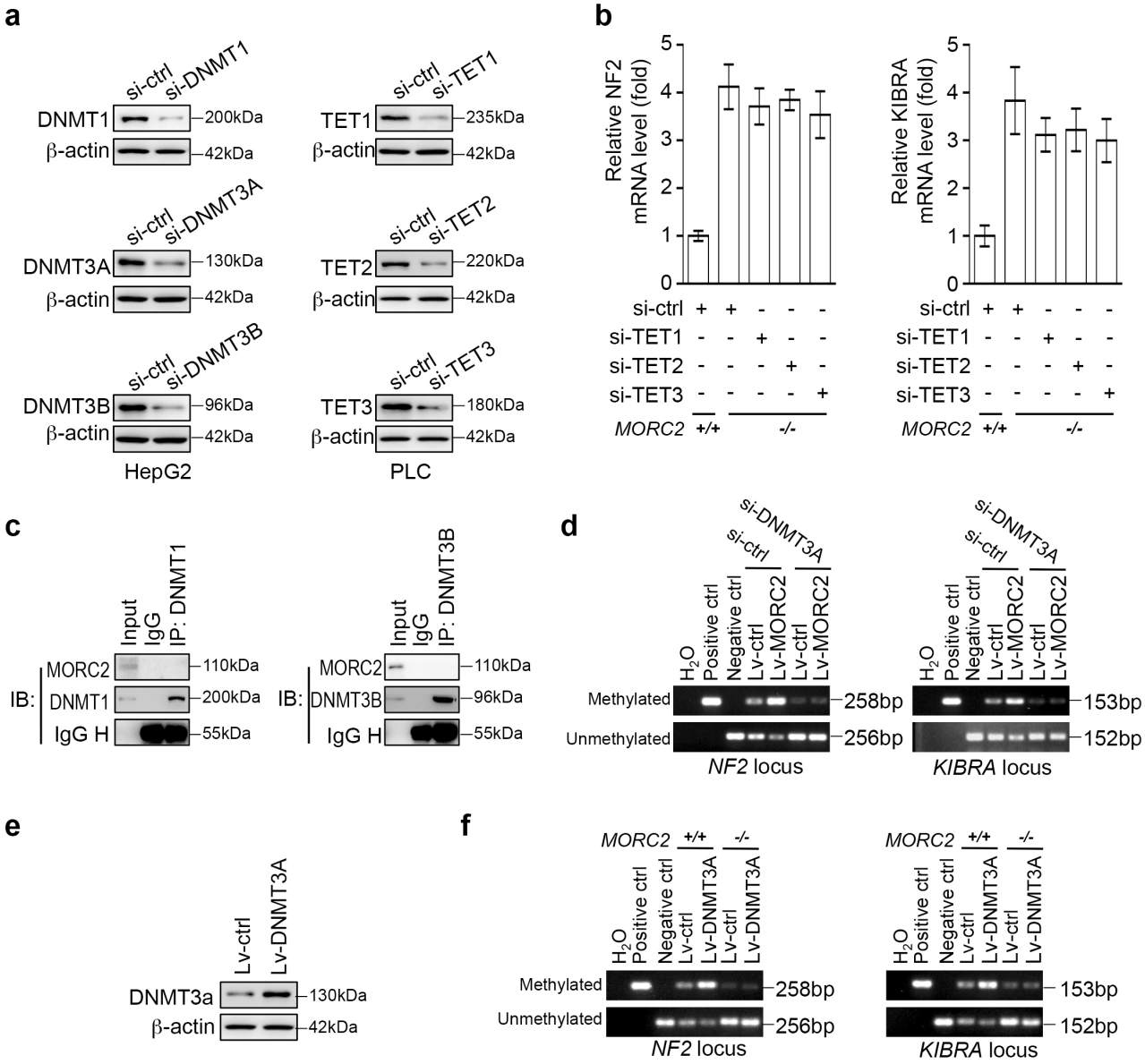
(**d, e**) The occupancy of indicated posttranslational histone modifications in the promoter regions of

NF2 and *KIBRA* loci was determined by ChIP-qPCR.

(**f, g)** MeDIP-qPCR analysis of DNA methylation status of the *NF2* and *KIBRA* loci in control or MORC2-expressing HepG2 cells, or various PLC cells (*MORC2*$^{+/+}$ or *MORC2*$^{-/-}$ or *MORC2*$^{-/-}$ cells expressing ectopic MORC2).

(**h)** The occupancy of MORC2 at the *NF2* and *KIBRA* promoters in HepG2 cells was examined by ChIP-PCR.

Normal IgG was included as negative control for ChIP-qPCR and MeDIP-qPCR. Data are presented as the mean ± SD from triplicate experiments (**a**, **b**, **d**-**g**). Representative images are presented in **h**. *, $P < 0.05$.



Supplementary Figure 3. The DNA methyltransferase DNMT3A is required for MORC2 to promote methylation at the *NF2* and *KIBRA* loci.

(a) HepG2 or PLC cells were transfected with scramble siRNA (si-ctrl) or siRNAs targeting indicated genes. The knockdown efficiency was confirmed by western blot analysis.

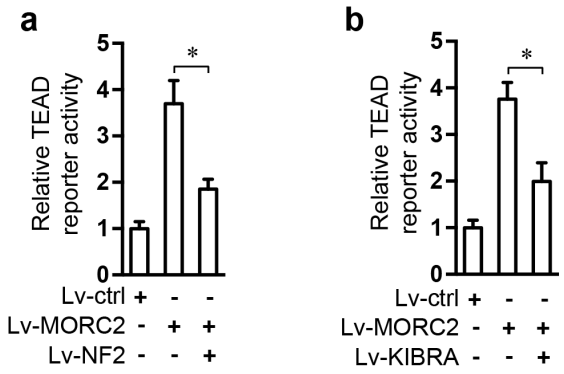
(**b)** Unlike DNMT3A, knockdown of TET1, TET2 or TET3 minimally affects mRNA levels of NF2 and KIBRA in *MORC2*$^{-/-}$ PLC cells. The mRNA levels of NF2 and KIBRA were determined by qRT-PCR analysis.

(c) Western blot analysis of anti-DNMT1 and anti-DNMT3b immunoprecipitates.

(**d-f**) MORC2 and DNMT3A depend on each other to promote DNA methylation of *NF2* and *KIBRA* loci. MSP analysis of DNA methylation status of *NF2* and *KIBRA* promoters in control or MORC2-expressing HepG2 that were transfected with scramble siRNA or si-DNMT3A, or in *MORC2*$^{+/+}$ or *MORC2*$^{-/-}$ PLC cells infected with control or DNMT3A-expressing lentivirus. Upper or lower gel images are obtained using amplicons with primer sets specific for

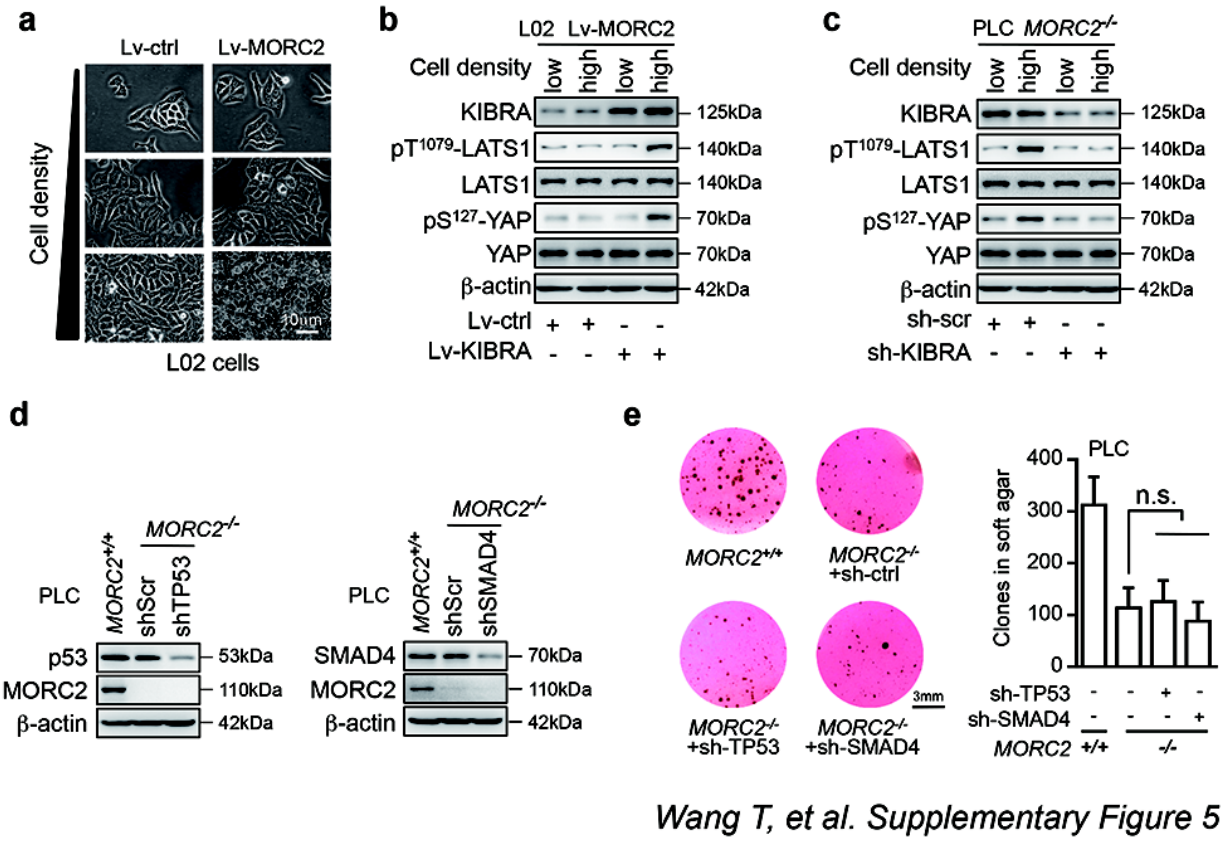
methylated or unmethylated sequences, respectively. *In vitro* methylated DNA from blood lymphocytes was used as positive control, with Na bisulfite untreated DNA as negative control, deionized water as no template control (**d**, **f**). Lentivirus-mediated ectopic expression of DNMT3A in PLC cells was validated by western blot analysis (**e**).

Representative images are shown (**a**, **c**-**f**). Data are presented as the mean ± SD of each group from triple replicates (**b**). *, $P < 0.05$.



Supplementary Figure 4. MORC2 enhances YAP transcriptional co-activation activity *via* suppressing NF2 and KIBRA expression.

(**a**, **b**) Control (Lv-ctrl) and MORC2-expressing (Lv-MORC2) HepG2 cells were infected with lentivirus encoding either NF2 (**a**) or KIBRA (**b**). The luciferase activities of 8x-GTIIC (TEAD) reporter in these cells were determined and presented as mean ± SD. * $P < 0.05$.



Wang T, et al. Supplementary Figure 5

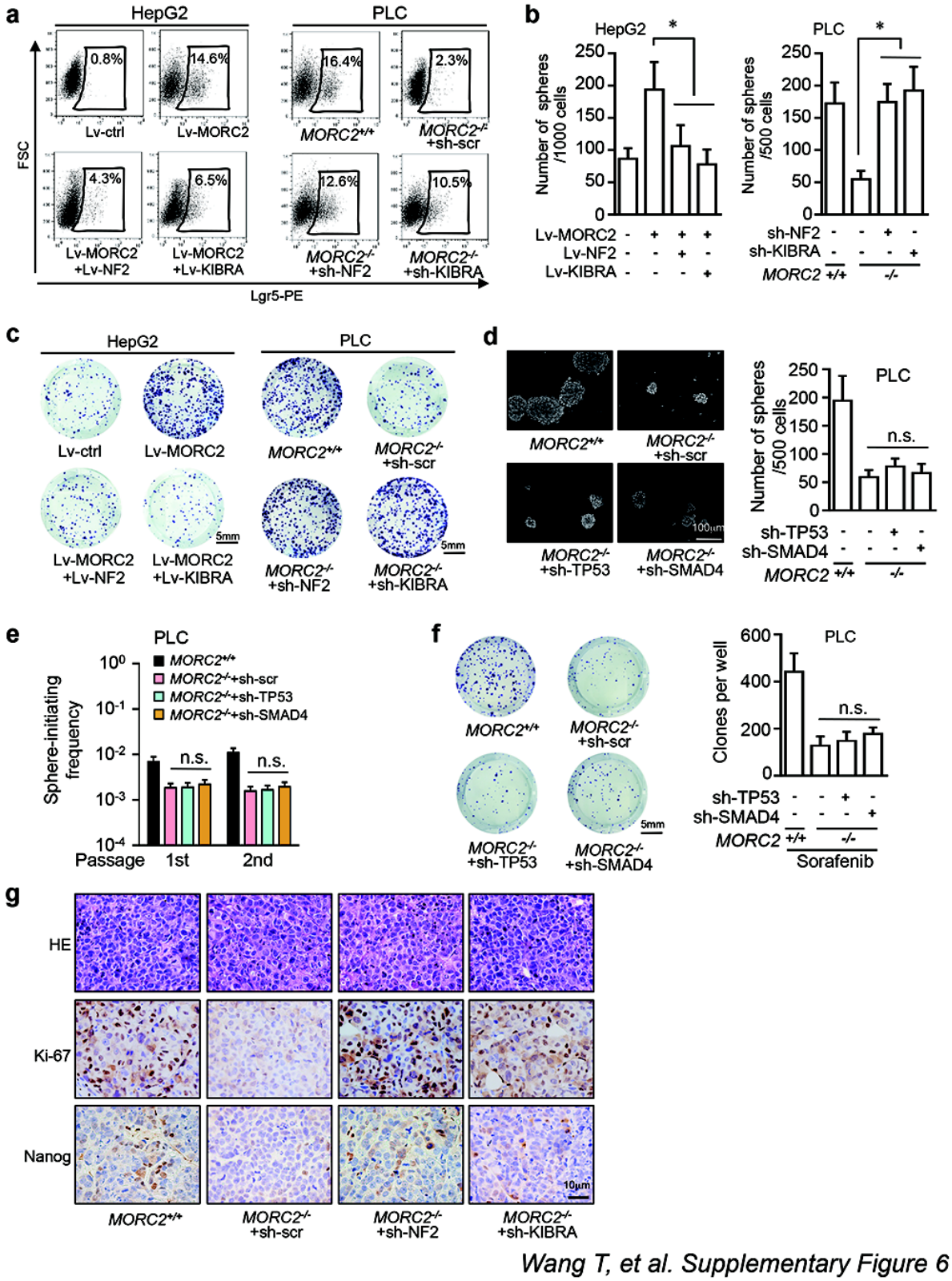
Supplementary Figure 5. The MORC2-NF2/KIBRA axis regulates confluence-induced activation of Hippo signaling and growth arrest.

(a) The different morphologies of control and MORC2-expressing L02 cells at low, intermediate or high confluence.

(b, c) MORC2-expressing L02 cells were infected with control or KIBRA-encoding lentivirus **(b)**. *MORC2*$^{-/-}$ PLC cells were infected with lentivirus delivering scramble shRNA or KIBRA-specific shRNA **(c)**. The activity of Hippo signaling in cells at low or high confluence was examined by western blot.

(d, e) *MORC2*$^{-/-}$ PLC cells were infected with lentivirus delivering scramble shRNA or shRNAs targeting indicated genes. Knockdown efficiency was confirmed by western blot analysis **(d)**. Indicated cells were used for soft agar colony formation assay (**e**).

Representative images from triplicate experiments are presented (**a**-e). * $P < 0.05$. Data are presented as the mean ± SD of each group from triple replicates (**e**). n.s., not significant.



Wang T, et al. Supplementary Figure 6

Supplementary Figure 6. The MORC2-NF2/KIBRA axis dictates self-renewal and tumorigenicity of HCC cells.

(a) Control and MORC2-expressing HepG2 cells were infected with empty vector or lentivirus encoding NF2 or KIBRA. *MORC2*$^{+/+}$ and *MORC2*$^{-/-}$ PLC cells were infected with lentivirus

delivering scramble shRNA, or NF- or KIBRA- specific shRNA. $Lgr5^{+}$ cancer stem cells were examined by flow cytometry and representative results were shown.

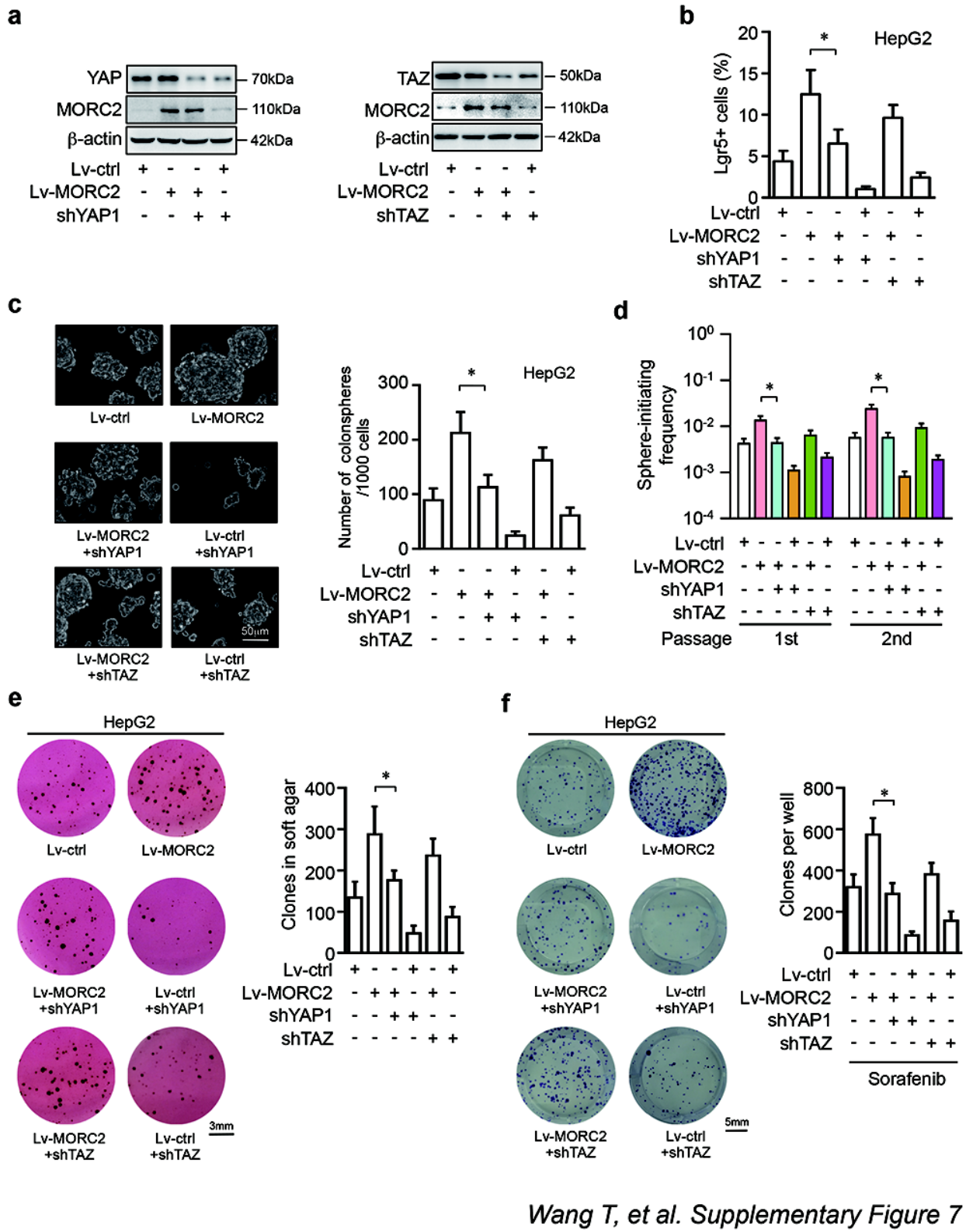
(**b**) HCC cells were treated as in (**a**) and single cells were seeded in stem cell medium to grow as tumorspheres. The numbers of tumorspheres generated by these cells were presented as the mean ± SD (* $P < 0.05$).

(**c**) Representative images of colonies grown from HCC cells as treated in (**a**) in the presence of Sorafenib.

(d-f) *MORC2*$^{-/-}$ PLC cells were infected with lentivirus delivering scramble shRNA or shRNAs targeting *TP53* or *SMAD4*. Cells were seeded in stem cell medium for tumorsphere assays. Representative images and the numbers of tumorspheres were shown in (**d**). The frequencies of sphere-initiating cells were determined by an extreme limiting dilution assay of serially passaged tumorspheres in stem cell medium. (tumorspheres with a diameter larger than 50 μm) (**e**). Alternatively, cells were grown in the presence of sorafenib for two days and the numbers of colonies were quantified **(f).**

(**g**) Representative images of HE staining and IHC staining for proliferation marker Ki-67 and stem cell marker Nanog in serial sections of xenograft tumors generated by indicated HCC cells.

Representative results **(a)** or images (**c, d, f, g**) are shown. Data are presented as the mean ± SD (**b, d, f**) or mean ± 95% confidence interval (**e**) of each group from triple replicate. *P<0.05. n.s., not significant.



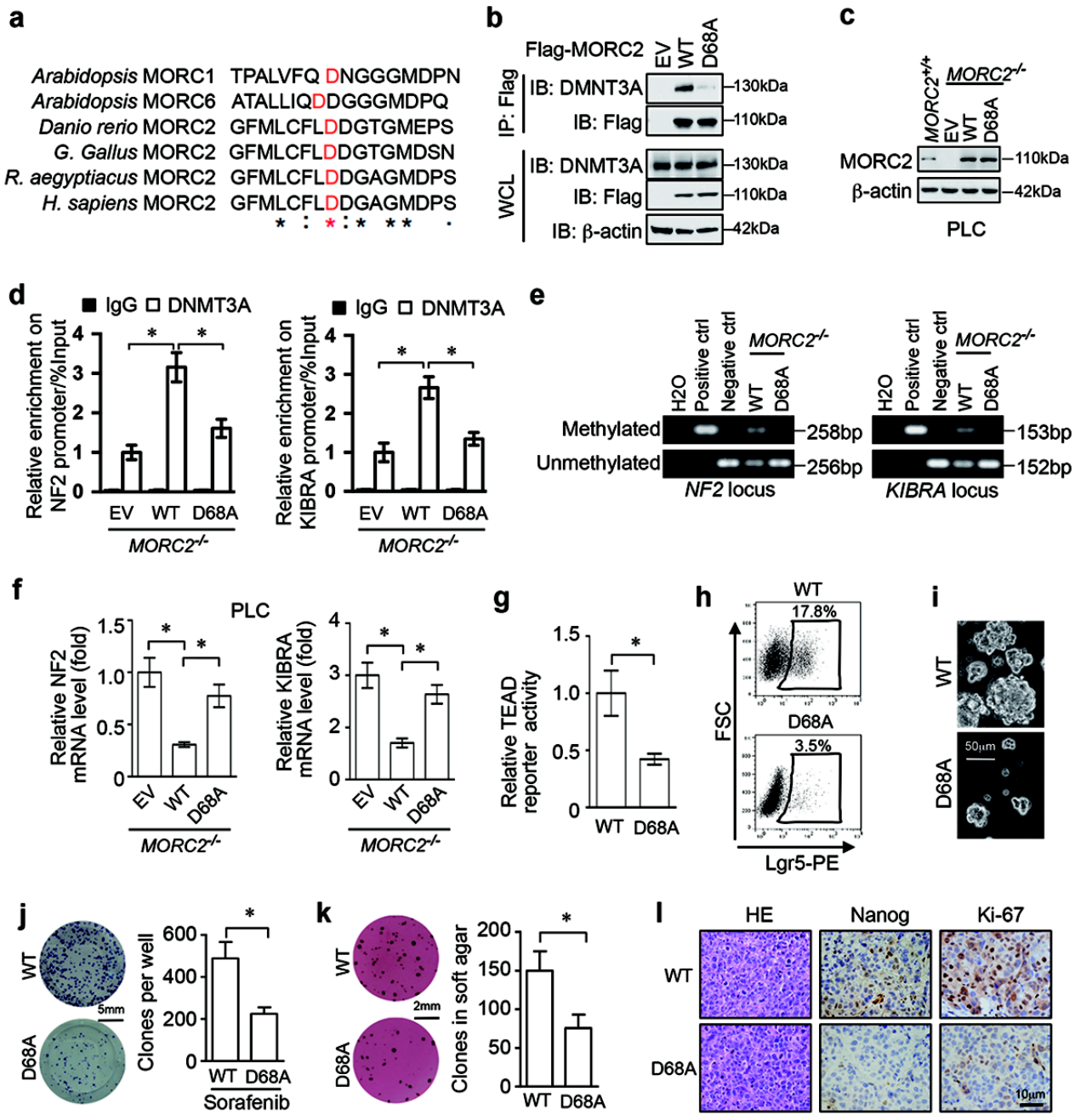
Wang T, et al. Supplementary Figure 7

Supplementary Figure 7. YAP1, and to a lesser extent TAZ, are downstream mediators of MORC2 to promote self-renewal of HCC cells.

(a-f) Control (Lv-ctrl) and MORC2-expressing (Lv-MORC2) HepG2 cells were infected with empty vectors or lentivirus delivering scramble shRNA, YAP1 or TAZ specific shRNA **(a)**. The percentages of Lgr5^{+} liver cancer stem cells were examine by flow cytometry **(b)**. Single cells were seeded in stem cell medium to grow as tumorspheres. Representative images and the

numbers of tumorspheres were shown (**c**). The single cells from primary and secondary tumorspheres were cultured in stem cell medium in a limiting dilution manner for 14 days and the frequencies of sphere-initiating cells were calculated (tumorspheres with a diameter larger than 50 μm) (**d**). The anchorage independent growth capacities of these cells were examined (**e**). Cells were treated with Sorafenib (4 μM) for 48 hours and cultured in normal medium for 10 days to examine colony formation capacity. Colonies with a diameter greater than 75 μm were counted. Representative images were shown (**f**).

Representative images are shown (**a, c, e, f**). Data are presented as the mean ± SD (**b, c, e, f**) or mean ± 95% confidence interval of each group from triple replicate (**d**). * $P < 0.05$.



Supplementary Figure 8. ATPase activity is essential for MORC2 to facilitate methylation of *NF2* and *KIBRA* loci, thereby regulating stemness and Sorafenib sensitivity.

(a) D68 of MORC2 is evolutionarily conserved. Alignment of amino acid sequence around the key catalytic residue D68 (Red) of GHKL-ATPase domain of human MORC2 and its homologues in multiple species. (* highly conserved, : moderately conserved, . low conservation).

(b) Western blot analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from HEK293 cells transfected with empty vector (EV), Flag-tagged MORC2-WT or MORC2-D68A.

(c) Western blot analysis validates generation of *MORC2*$^{-/-}$ PLC cells by CRISPR/Cas9

gene-editing technology and *MORC2*$^{-/-}$ PLC cells that were stably re-introduced with MORC2-WT or MORC2-D68A by lentivirus infection.

(d) ATPase activity of MORC2 is required for DNMT3A to efficiently localize at *NF2* and *KIBRA* loci. The occupancy of DNMT3A at the *NF2* and *KIBRA* promoters was determined by ChIP-qPCR. Normal IgG was included as negative control.

(**e**) ATPase-deficient MORC2 cannot efficiently promote DNA methylation of *NF2* and *KIBRA* loci. MSP analysis of DNA methylation status of *NF2* and *KIBRA* promoters in *MORC2*$^{-/-}$ PLC cells expressing MORC2-WT or MORC2-D68A. *In vitro* methylated DNA from blood lymphocytes was used as positive control, with Na bisulfite untreated DNA as negative control, deionized water as no template control.

(f) ATPase-deficient MORC2 fails to suppress expression of NF2 and KIBRA. The relative levels of NF2 and KIBRA mRNA were determined by qRT-PCR (n=3 per biological repeats).

(g) ATPase-deficient MORC2 fails to promote YAP transcriptional co-activator activity. The levels of luciferase activity of 8x-GTIIC (TEAD) reporter were detected in *MORC2*$^{-/-}$ PLC cells expressing MORC2-WT or MORC2-D68A.

(h-l) ATPase activity of MORC2 is critical for maintaining a pool of Lgr5^{+} CSCs, tumorsphere growth, sorafenib resistance and tumorigenicity of HCC cells. *MORC2*$^{-/-}$ PLC cells were infected with lentivirus expressing MORC2-WT or MORC2-D68A. The percentages of Lgr5^{+} CSCs were examined by flow cytometry **(h)** and representative images of tumorsphere growth were monitored by microscopy **(i)**. The cells were grown in the presence of sorafenib for two days and the numbers of colonies were quantified **(j)**. The tumorigenicity of these cells was examined by colony formation in anchorage-independent cultures **(k)**. The xenograft tumors were dissected and representative IHC staining images displayed expression of proliferation marker Ki-67 and stem cell marker nanog in serial tissue sections **(l)**.

Representative images are shown (**b**, **c**, **e**, **h-l**). Data are presented as the mean ± SD (**d**, **f**, **g**, **j**, **k**) of each group from triple replicate. * $P < 0.05$.