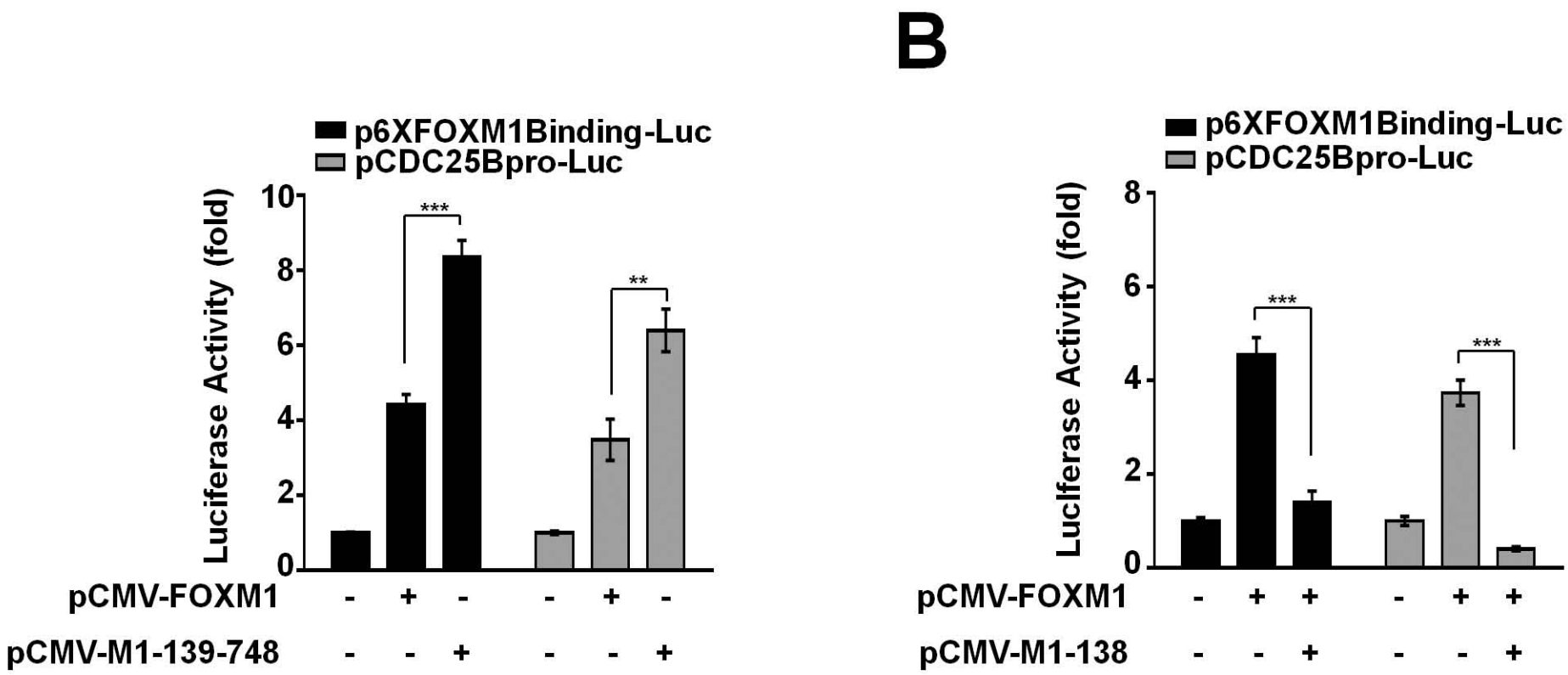
The cell-penetrating FOXM1 N-terminus (M1-138) demonstrates potent inhibitory effects on cancer cells by targeting FOXM1 and FOXM1-interacting factor SMAD3

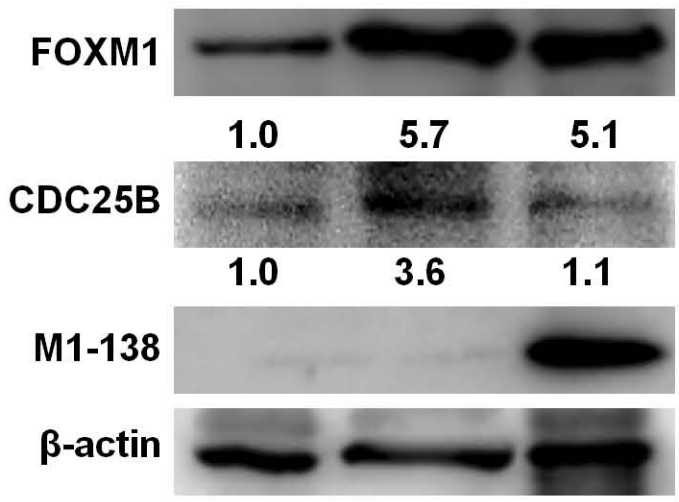
Running title: Protein-based inhibitor of FOXM1 in cancer cells

Zhenwang Zhang, Huitong Bu, Jingwei Yu, Yan Chen, Chaozhu Pei, Li Yu, Xiaoqin Huang, Guixiang Tan* and Yongjun Tan*

Supplementary Figures

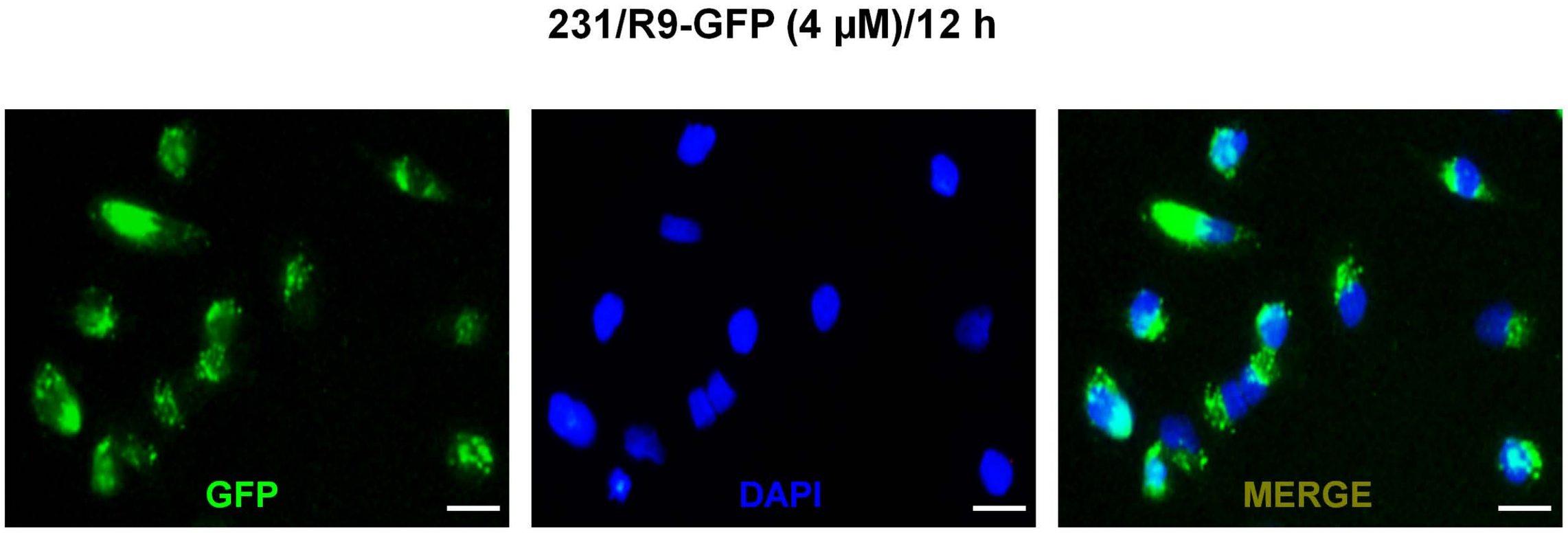
Figure S1





The N-terminus of FOXM1 protein acted as an inhibitory domain to FOXM1 transcriptional activities. A, A FOXM1 expression vector (pCMV-FOXM1, 1 μg) or a N-terminus-deleted FOXM1 expression vector (pCMV-M1-139-748, 1 μg) was transfected with a luciferase reporter plasmid containing 6x FOXM1 binding sequences in its promoter (p6xFOXM1Binding-Luc, 1 μg) or a −2.3 kb CDC25B promoter-luciferase reporter plasmid (pCDC25Bpro-Luc, 1 μg) plus loading control pRL-CMV luciferase reporter plasmid (20 ng) into HEK293T cells. Protein lysates were prepared at 48 h later and used for the measurement of dual Luciferase activities. B, The pCMV-FOXM1 (1 μg) was transfected into HEK293T cells with p6xFOXM1Binding-Luc (1 μg) or pCDC25Bpro-Luc (1 μg), plus or not a FOXM1 N-terminus expression vector (pCMV-M1-1-138, 1 μg). Protein lysates were prepared at 48 h post transfection and used for the measurement of dual Luciferase activities. Each bar represents the mean Luciferase Activity ± SD (**, $P \leq 0.01$; ***, $P \leq 0.001$). C, The pCMV-FOXM1 (1 μg) was cotransfected with pCMV-M1-138 (1 μg) and 48 h later the cells were harvested for the preparation of total proteins. The protein levels of FOXM1, CDC25B, and M1-138 in the cells were examined by Western blotting. The detection of β-actin was performed as loading controls.

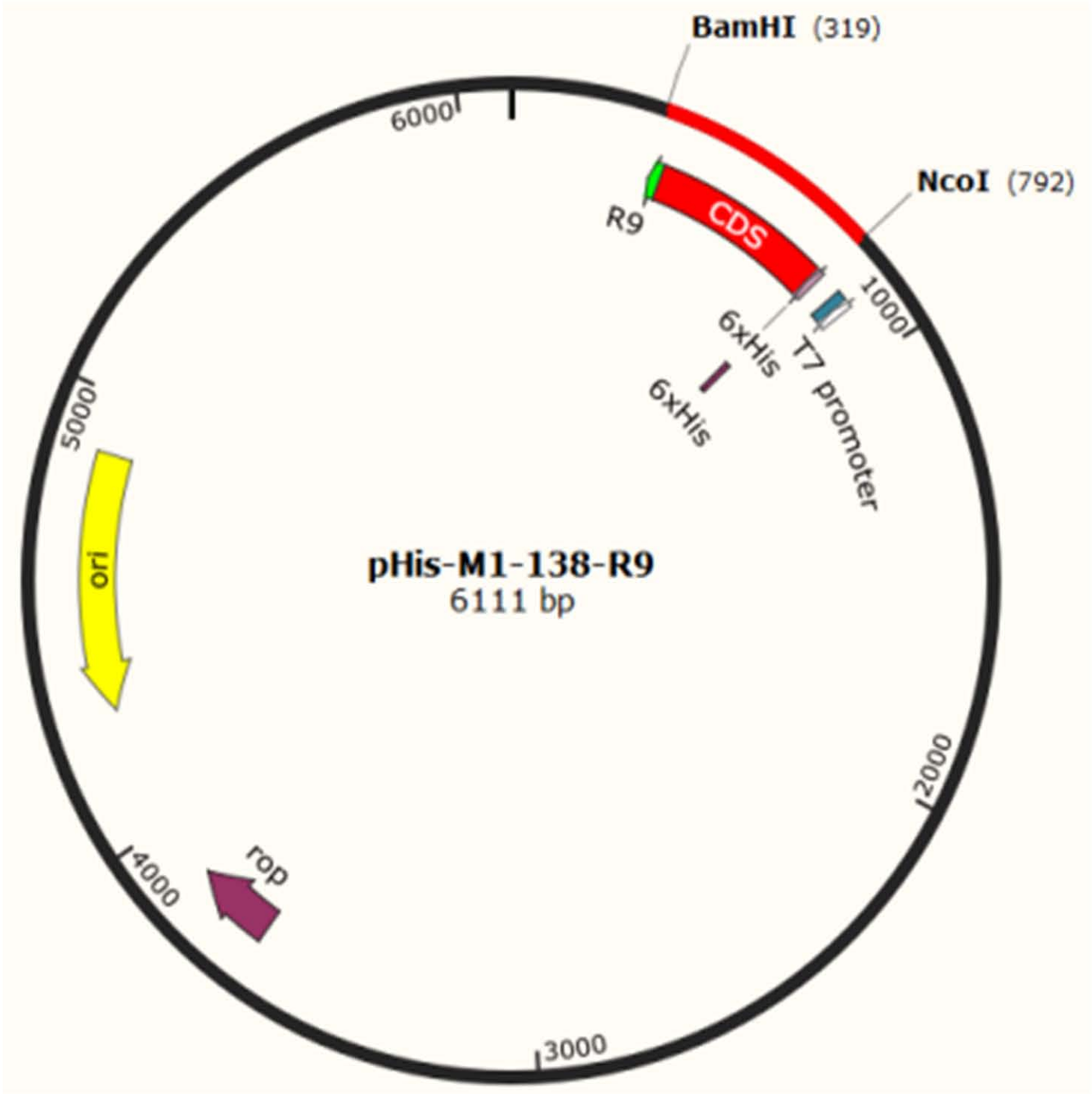
Figure S2



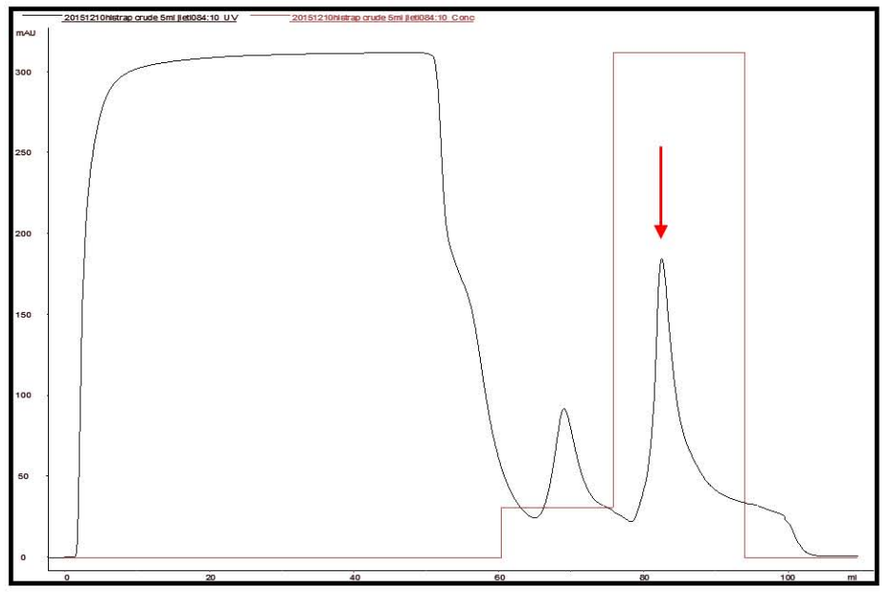
The R9 cell penetrating peptide-fused GFP entered cancer cells. MDA-MB-231 cells was incubated with R9-GFP (4 μM) for 12 h. The signals of GFP in cells were imaged with the fluorescent Con-focal microscope (Olympus FluoView FV1000). DAPI staining showed the location of nuclei. (Scale bars: 20 μm).

Figure S3

A



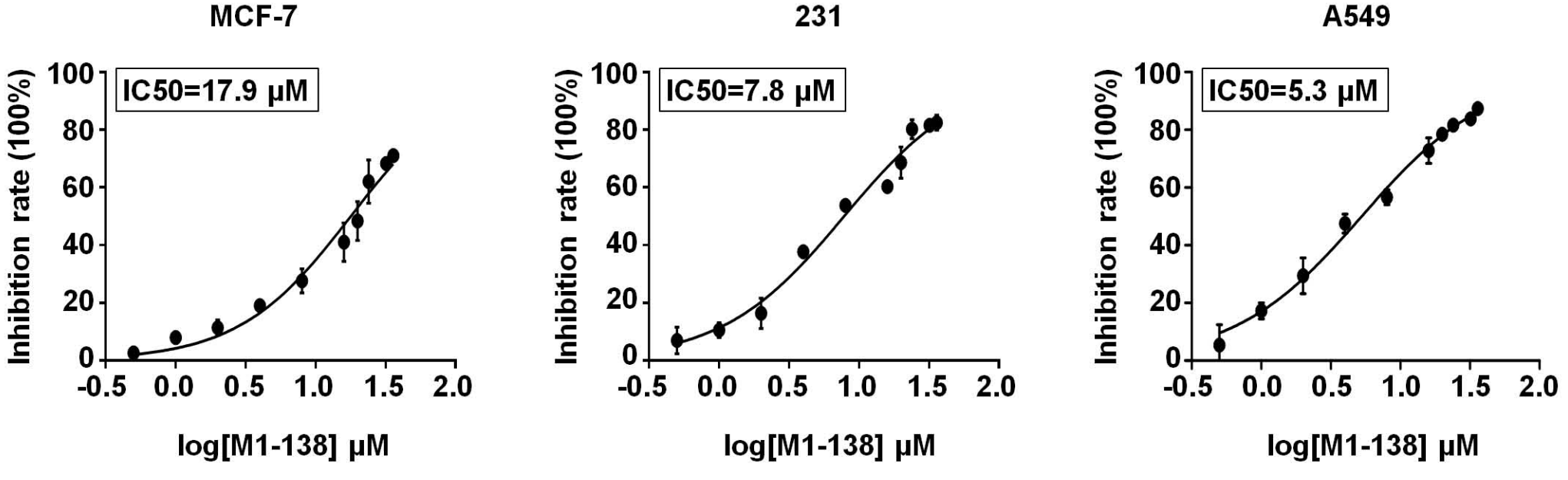
B



The production of recombinant M1-138. A, The construction map of a prokaryotic expression plasmid pHis-M1-138-R9. For the plasmid construction, a DNA fragment containing a 6xHis tag sequence (6xHis), FOXM1 N-terminus 1-138aa coding region (CDS), and the R9 peptide sequence (R9) was ligated into the pET-15b vector at NcoI and BamHI restriction sites. B, pHis-M1-138-R9 was transformed into Rosetta/DE3 *E. Coli* cells and the recombinant M1-138 was prepared according the procedure described in Materials and Methods. An AKTA Protein Purifier with a Ni-NTA agarose affinity chromatography column was used to purify M1-138 from *E. coli* cell lysates. The absorbance at 280 nm of the purification process was recorded during gradient washing and elution of M1-138. The absorption peak of collected M1-138 was marked by a red arrow. An SDS-PAGE electrophoresis was performed to analyze the samples from the different stages of the purification process. A red arrow pointed the position of the purified M1-138.

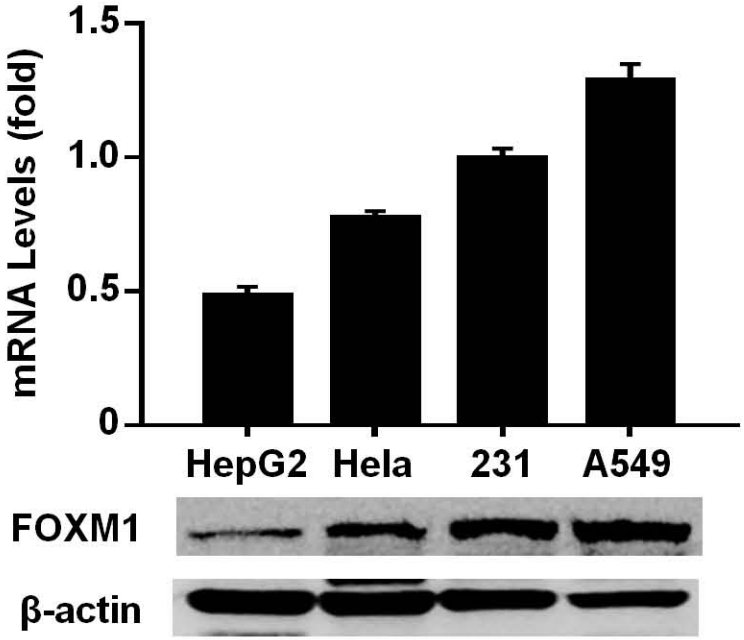
Figure S4

A



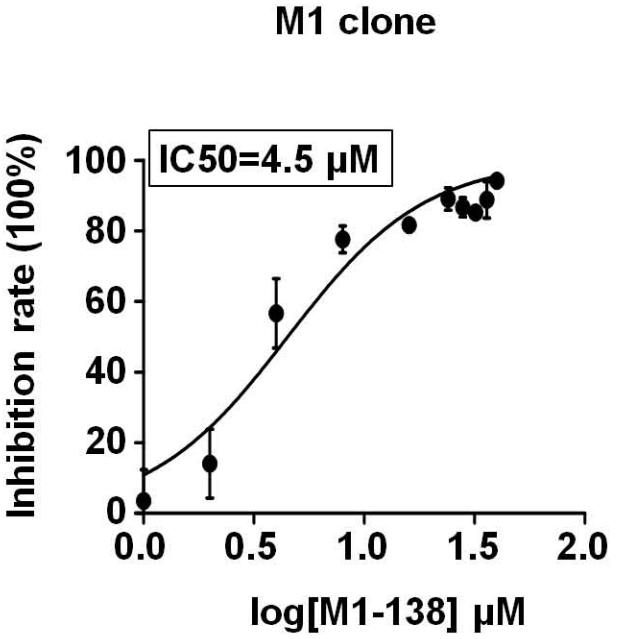
The calculation of the half maximal inhibitory concentration (IC50) of M1-138 for the tested cell lines. MCF-7, MDA-MB-231, A549, HepG2, and Hela cells were treated with increasing concentrations of M1-138 (0.5, 1, 2, 4, 8, 16, 20, 24, 32, 36 μM) for 12 h. The cell viability was measured by CCK-8 assays. The inhibition rate (IR) in response to M1-138 at different doses was calculated according to the equation: IR=(1- OD_{450} of M1-138/OD_{450} of R9-GFP) ×100%. With GraphPad software, the logarithm (log [M1-138]) of the M1-138 doses was plotted against IR and the IC50 value for each cell line was calculated. Values represented the mean ± SD of four replicates.

Figure S5



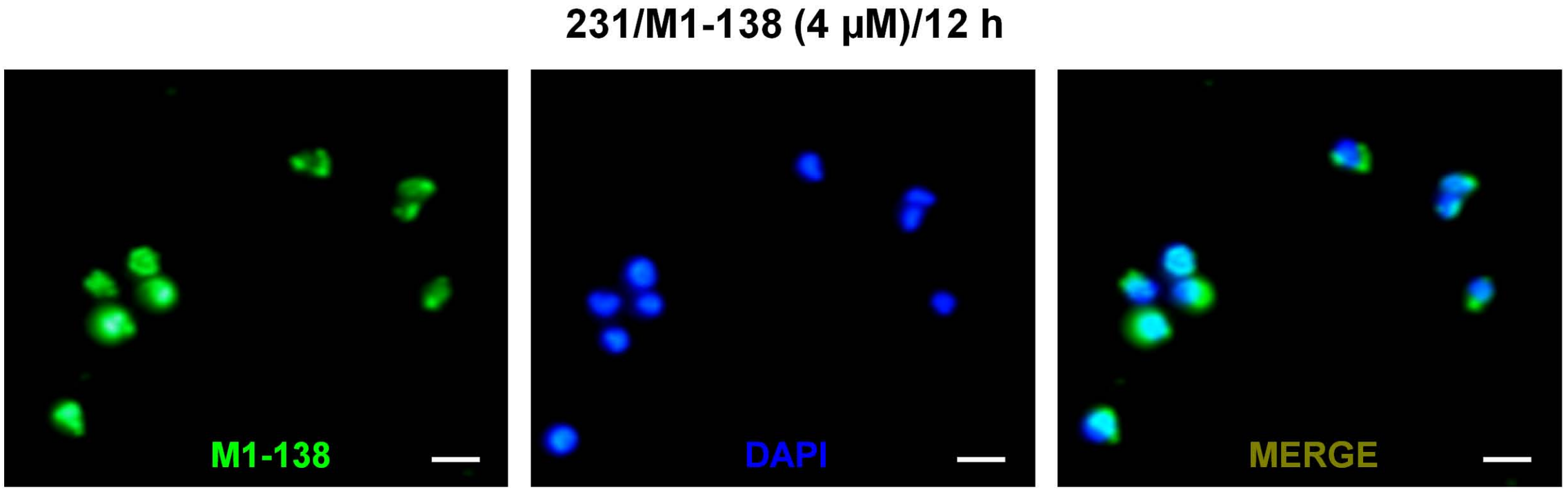
The expression levels of FOXM1 in cancer cells. HepG2, Hela, MBA-MD-231 (231), and A549 cells were harvested to prepare total RNAs and protein lysates. The mRNA levels of FOXM1 were measured by qPCR and the protein levels of FOXM1 were measured by Western blotting. The detection of GAPDH mRNA and β-actin protein were performed as loading controls for RNAs and protein lysates respectively. Relative mRNA levels were normalized to GAPDH. The levels of MDA-MB-231 cells were referred as One.

Figure S6



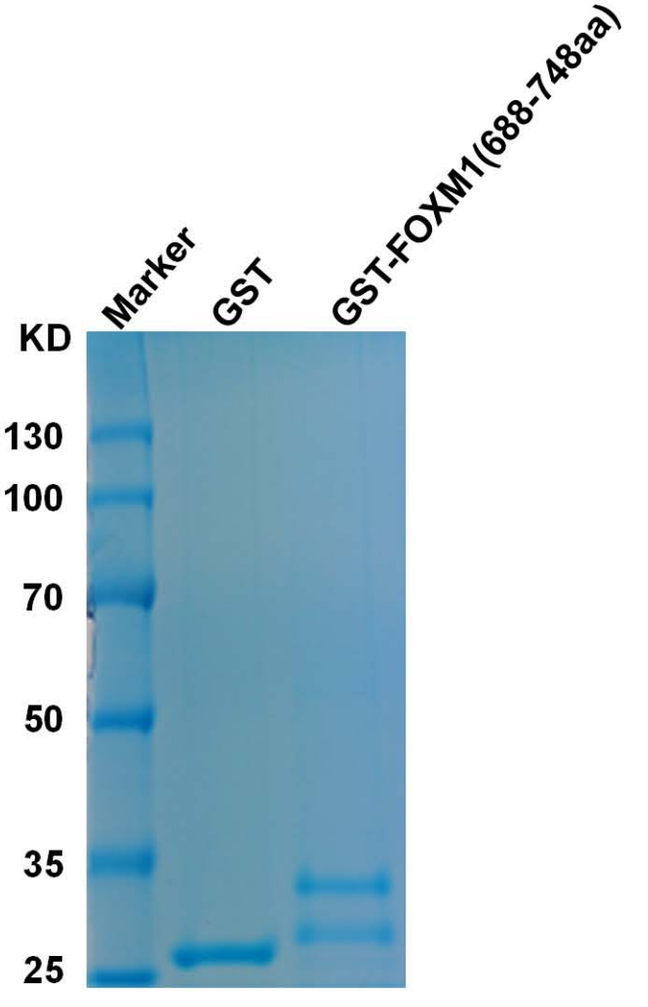
The calculation of IC50 of M1-138 for MCF-7 M1 clone cells. M1 clone cells was treated with increasing concentrations of M1-138 (0.5, 1, 2, 4, 8, 16, 20, 24, 32, 36 μM) for 12 h. The cell viability was measured by CCK-8 assays. The inhibition rate (IR) in response to M1-138 at different doses was calculated according to the equation: IR= (1 - OD_{450} of M1-138/OD_{450} of R9-GFP) ×100%. With GraphPad software, the logarithm (log [M1-138]) of the M1-138 doses was plotted against IR and the IC50 value for M1 clone was calculated. Values represented the mean ± SD of four replicates.

Figure S7



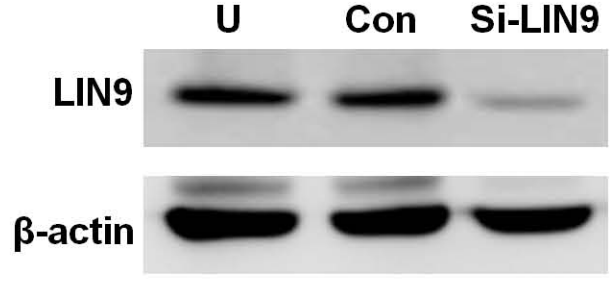
M1-138 distributed in both cytoplasm and nucleus of MBA-MD-231 cells. MDA-MB-231 cells (1×10^4 cells) were seeded in a 35-mm glass bottom petri dish for 24 h and treated with M1-138 (4 μM). 12 h later, the cells were fixed with 4% formaldehyde for 20 min at room temperature and blocked with 10% bovine serum albumin (BSA) for 15 min. Afterwards, the cells were incubated with rabbit anti-His antibody at 4 ° C overnight and followed by the incubation of fluorescent AlexaFlour 488-conjugated anti-rabbit IgG. The cells were imaged with the fluorescent Con-focal microscope (Olympus FluoView FV1000). DAPI staining showed the location of nuclei. (Scale bars: 20 μm).

Figure S8



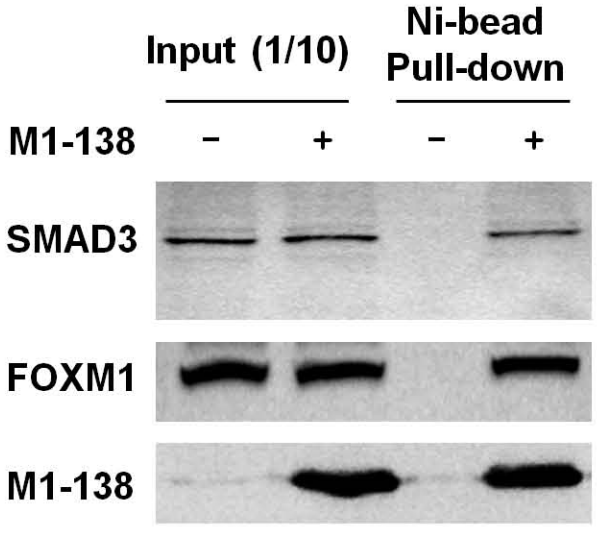
SDS-PAGE electrophoresis of purified recombinant GST-FOXM1(688-748aa). pGST-FOXM1(688-748aa) was transformed into Rosetta/DE3 *E. Coli* cells and the recombinant GST-FOXM1(688-748aa) was purified according the procedure described in Materials and Methods. An SDS-PAGE gel electrophoresis was performed to analyze the purified samples of GST (left) and GST-FOXM1(688-748aa) (right).

Figure S9



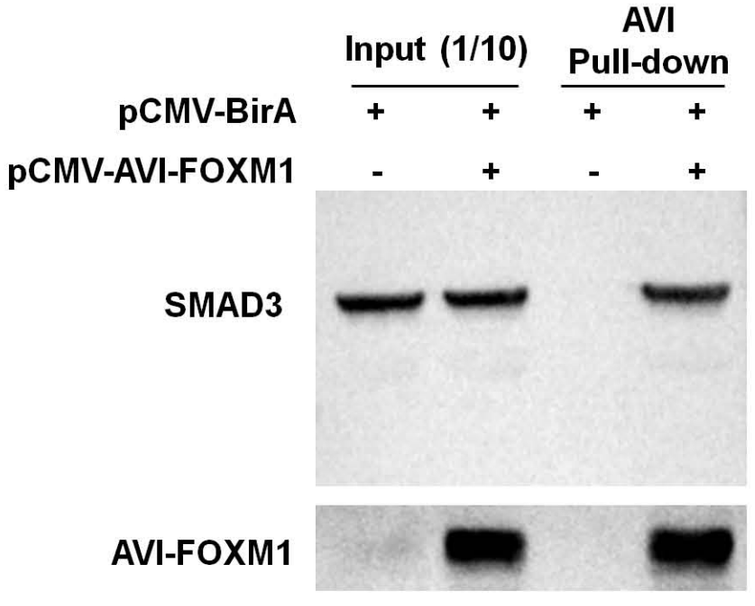
LIN9 siRNA to abolish the LIN9 expression. Hela cells were transfected with control siRNA (Con), LIN9 siRNAs, or left untreated (U). The expression of LIN9 and β-actin in whole-cell lysates was detected by Western blotting.

Figure S10



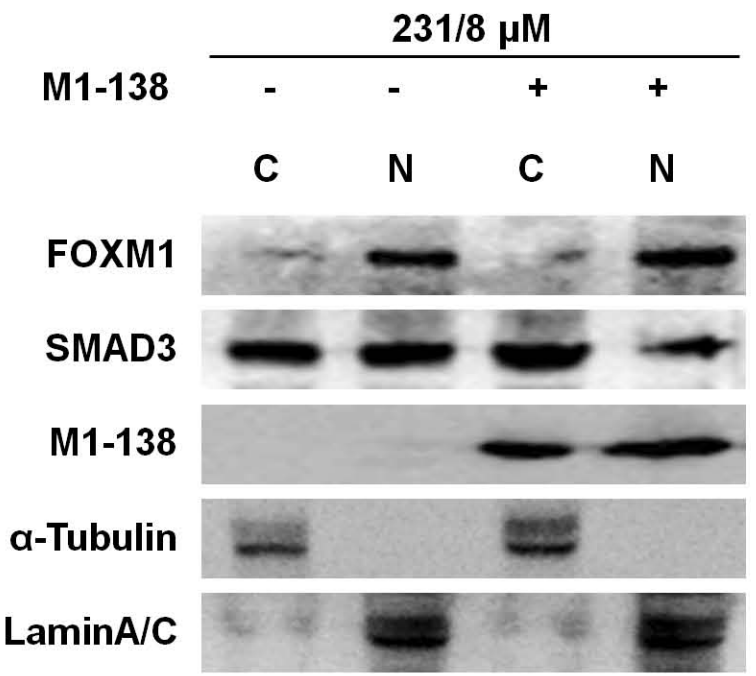
M1-138 bound to FOXM1 and SMAD3 in Hela cells. The lysates (500 μg) of M1-138-treated Hela cells were incubated with Ni-NTA agarose beads to pull down His-tagged M1-138/protein complexes. SMAD3, FOXM1, and M1-138 in the pull-down samples were detected by Western blotting with anti-SMAD3 antibody or anti-FOXM1 antibodies. Fifty μg lysates (10% of input) was also used for Western blotting as controls.

Figure S11



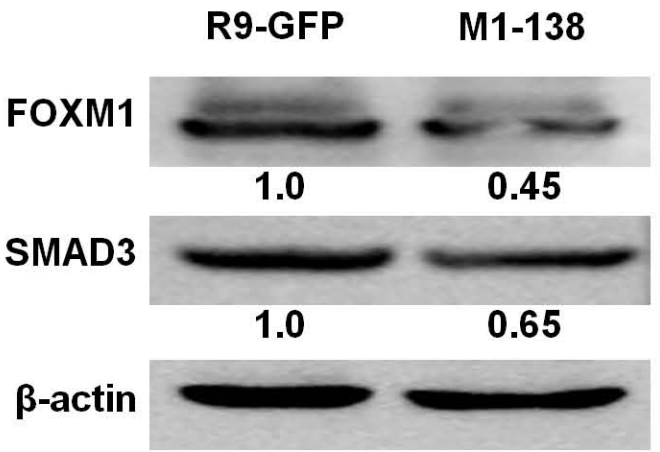
FOXM1 bound to SMAD3. HEK293T cells were transfected with pCMV-BirA or pCMV-AVI-FOXM1 and pCMV-BirA and 48 h later the cell lysates were prepared. The lysates (500 μg) were incubated with Streptavidin agarose beads to pull down AVI-FOXM1/protein complexes. SMAD3 and AVI-FOXM1 proteins in the pull-down samples were detected by Western blotting with anti-SMAD3 antibody or HRP-labeled Streptavidin. 10% of the reactions (50 μg) was also used for Western blotting as input controls.

Figure S12



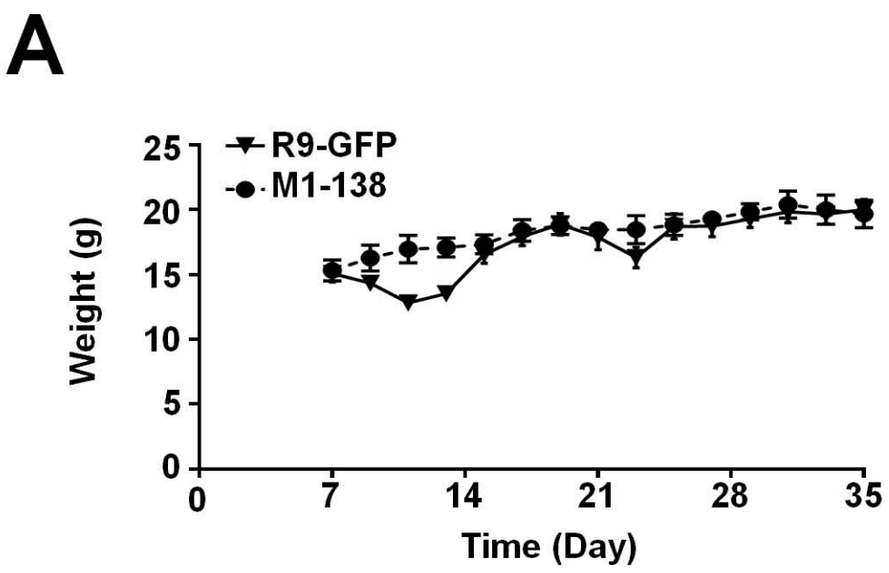
The localization of FOXM1 and SMAD3 was detected by Western blotting. MDA-MB-231 cells (2×10^5 cells/well) were seeded in 6-well plates for 24 h and then treated with M1-138 (8 μM). 12 h later, the cells were harvested for the preparation of cytoplasmic and nuclear proteins. The levels of FOXM1, SMAD3, and M1-138 in cytoplasm (C) or nucleus (N) were examined by Western blotting. The levels of α-Tubulin or LaminA/C were also detected as cytoplasmic or nuclear markers, respectively.

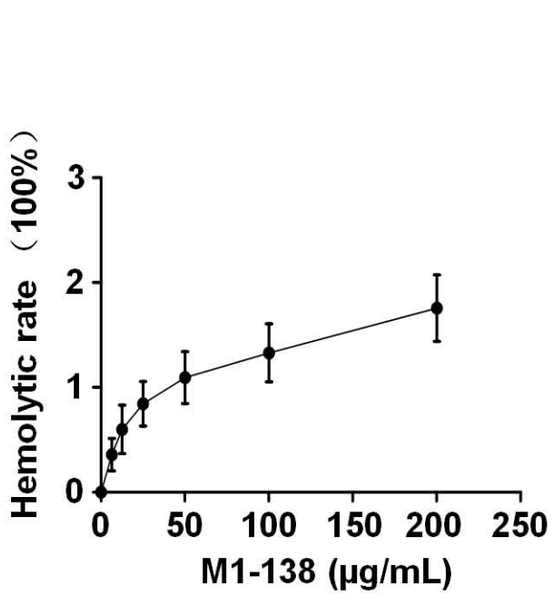
Figure S13

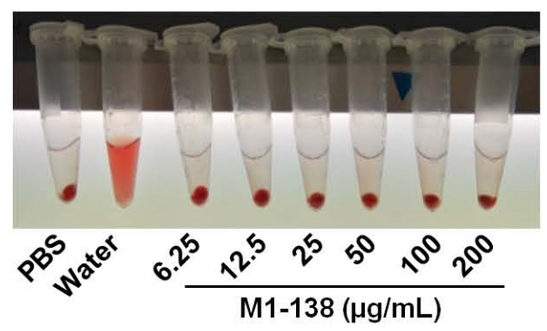


The protein levels of FOXM1 and SMAD3 were down-regulated in M1-138-treated engrafted tumors. Total proteins were prepared with the harvested tumors and the pools of combined protein lysates for the R9-GFP-treated or M1-138-treated groups were obtained. The protein levels of FOXM1 and SMAD3 were measured by Western blotting. The detection of β-actin was performed as loading controls.

Figure S14







C

M1-138 toxicity test

	14 day	14 day	14 day	14 day
M1-138 (mg/kg)	20	40	60	180
Dead/Alive	0/3	0/3	0/3	0/3

M1-138 was well tolerated *in vivo*. A, During the period of testing M1-138 effects on tumor growth *in vivo* (4 mg/Kg/day×14 days), the body weight of tested animals was measured once every two days post the treatment and the growth curves of mice in the two groups were obtained. B, A 10% (v/v) suspension (100 μL per sample) of ICR/JCL mouse erythrocytes in normal saline (NS, 0.9%) were treated with deferent concentrations of M1-138 (6.5, 12.5, 25, 50, 100, 200 μg/mL) for 3 h at 37 ° C. The sterile double distilled water or PBS (100 μL) was set up as positive or negative controls respectively. After centrifugation at 1200 g for 15 min at 4 ° C, 100 μL supernatant was moved into a 96-well plate and the absorbance was measured at 540 nm using a microplate reader. The hemolytic rate (IR) in response to M1-138 at different doses was calculated according to the equation: IR % = [(ODsample - ODnegative) / (ODpositive - ODnegative)] × 100%. The percentage of IR versus the concentration of M1-138 was plotted. The picture was taken for the test tubes at the end of the experiments. C, ICR/JCL mice (6 weeks old) were randomized into four groups (three mice per group) and intraperitoneally injected with different doses of M1-138 (20, 40, 60, and 180 mg/Kg body weight). The mice were continuously observed for diet and activity for 14 days.

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Running title: Protein-based inhibitor of FOXM1 in cancer cells

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Supplementary Materials and Methods

Construction of plasmids

The cDNA of FOXM1 N-terminus (1-138aa) was PCR amplified from pCMV-FOXM1 plasmid (1) with primers containing NcoI and BamHI restriction sites, 6xHis-tag, and nine arginine cell-penetrating peptide（R9）(sense primers: 5'-GCG CCC ATG GTG CAT CAC CAT CAC CAT CAC ATG AAA ACT AGC CCC CGT CG-3' and antisense primers: 5'-GCG GAT CCC TAC CTT CTC CTT CTC CTT CTC CTT CTC CTC AGG GTC ACT TCT GTC-3') and ligated into the pET-15b vector (Novagen) to obtain the plasmid pHis-M1-138-R9. The cDNA of EGFP was PCR amplified from pEGFP-C2 plasmid (Addgene) with primers containing NcoI and Nde1 restriction sites, 6xHis-tag, and R9 (sense primers: 5'-GCG CCC ATG GTG CAT CAC CAT CAC CAT CAC ATG GTG AGC AAG GGC GA-3' and antisense primers: 5'-GCG CCA TAT GCT ACC TTC TCC TTC TCC TTC TCC TTC TCC TGA TCC GGT GGA TCC CGG-3') and ligated into the pET-15b vector to

obtain the plasmid pHis-GFP-R9. The cDNA of FOXM1 was PCR amplified from pCMV-FOXM1 plasmid with primers containing EcoRI and HindIII restriction sites, and AVI-tag (sense primers: 5'-GGA ATT CAT GTC CGG CCT GAA CGA CAT CTT CGA GGC TCA GAA AAT CGA ATG GCA CGA AAC TAG TAT GAA AAC TAG CCC CCG TCG-3' and antisense primers: 5'-CCC AAG CTT CTA CTG TAG CTC AGG AAT-3') and ligated into the pcDNA-3.1 vector (Thermo Fisher Scientific) to obtain the plasmid pCMV-AVI-FOXM1. The cDNA of FOXM1 N-terminus (1-138aa) was PCR amplified from pCMV-FOXM1 plasmid with primers containing XbaI and EcoRI restriction sites (sense primers: 5'-GCT CTA GAA TGA AAA CTA GCC CCC GTC GGC-3' and antisense primers: 5'-GGA ATT CTA ACA GGG TCA CTT CTG TC-3') and ligated into the pcDNA-3.1 vector to obtain the plasmid pCMV-M1-138. The cDNA of FOXM1 C-terminus (139-748aa) was PCR amplified from pCMV-FOXM1 plasmid with primers containing XhoI and HindIII restriction sites (sense primers: 5'-GCC TCG AGA TGG AGA CCT TGG GAC CAA AAC C -3' and antisense primers: 5'- GCA AGC TTC TAC TGT AGC TCA GGA ATA AAC-3') and ligated into the pcDNA-3.1(-) vector to obtain the plasmid pCMV-M1-139-748. SMAD3 cDNA was PCR amplified from a cDNA library of MDA-MB-231 cells with primers containing EcoRI and BamHI restriction sites (sense primers: 5'-CGG AAT TCC GAT GTC GTC CAT CCT GCC-3' and antisense primers: 5'-CGG GAT CCC TAA GAC ACA CTG GAA CAG CGG-3') and ligated into the pDsRed2-C1 vector (Addgene) to obtain the plasmid pCMV-SMAD3-RFP. The pHis-FOXM1 prokaryotic expression vector, pCMV-FOXM1-GFP, p6xFOXM1Binding-Luc and pCDC25pro-Luc reporter plasmids were described previously (2). The -1.4 kb PLK1 promoter was PCR amplified from human

genomic DNA with primers containing NheI and XhoI restriction sites (sense primers: 5'-CGG CTA GCC GCA CTA TAG GGG TAC-3' and antisense primers: 5'-CCC TCG AGG GGC AGA CCT CGA TCC G -3') and ligated into the pGL3-basic vector to obtain the plasmid pPLK1pro(-1.4 kb)-Luc. The -1.8 kb CDC25B promoter was PCR amplified from pCDC25pro-Luc plasmids with primers containing NheI and XhoI restriction sites (sense primers: 5'-CGG CTA GCC GGG TTT TTG GGC TGG G-3' and antisense primers: 5'-CCC TCG AGG GCT AGT TGC AGC TGC C-3') and ligated into the pGL3-Basic vector to obtain the plasmid pCDC25Bpro(-1.8 kb)-Luc. The pSMAD3Binding-Luc reporter plasmid was purchased from Genomeditech Co. Ltd. The pCMV-BirA vector was a gift from Dr. Xiaohua Shen of Tsinghua University, China.

CCK−8 cell viability assays

Cells were seeded in a 96-well plate (4×10^3 cells/well) and incubated for 12 h with 10% fetal bovine serum in a 5% CO_2-humidified atmosphere at 37 ℃. To measure the cell viability post treatments, 10 μL CCK-8 solution (Sigma-Aldrich) was added to each well and incubated for 2 h. The absorbance was measured at 450 nm by a Synergy HTX microplate reader (Biotek). The relative cell viability versus the treatement was plotted and IC50 was calculated by GraphPad software. The tests were performed in four repetitions.

Colony formation assays

Cells were seeded in a 6-well plate (400 cells/dish) and incubated for 24 h at 37 ℃, followed by M1-138 treatments. The cells were replenished every three days with fresh medium. After incubation for 14 days, the cells were fixed with 4% paraformaldehyde, rinsed three times with PBS, stained with 0.1% crystal violet for 10 min, and finally washed three

times with PBS. Pictures of the colonies were taken by a digital camera.

Transwell migration assays

The assay was performed using a 24-well plate Transwell with pore size of 8 μm (Corning). Cells treated with M1-138 for 12 h were collected and resuspended in serum-free medium. Subsequently, 1×10^5 cells were seeded in a top chamber of 200 μl of serum-free DMEM, and 600 μl of DMEM containing 10% FBS was applied to the lower chamber. After incubation at 37 ℃ for 24 h in a humidified environment of 5% CO_2, the cells on the upper membrane were gently removed by using a sterile cotton swab, and then washed three times with PBS. The cells on the inferior membrane were fixed with 4% paraformaldehyde for 20 min, washed twice with PBS, stained with 0.1% crystal violet for 10 min, and finally washed three times with PBS. Cells passing through the membrane were captured by a SMZ1000 light microscope (Nikon). The average cell counts were calculated from five random fields. The experiments were repeated three times.

Quantitative real−time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Omega) according to the manufacturer's instructions. Total RNA (2.0 μg) was reverse transcribed into 20 μl cDNA by RevertAid First Strand Kit (Promega). The qPCR was performed with SYBR Green (Toyobo) with following sense (S) and antisense (AS) primers: hFOXM1-S, 5’-GCT TGC CAG AGT CCT TTT TGC-3’ and hFOXM1-AS, 5'-CCA CCT GAG TTC TCG TCA ATG C-3’; hSMAD3-S, 5’-GGA GAA ATG GTG CGA GAA GG-3’ and hSMAD3-AS, 5’-GAA GGC GAA CTC ACA CAG C-3’; hCDC25B-S, 5’-AGT CCT GAC CGG AAG ATG GA-3’ and hCDC25B-AS, 5’-GAT GTT GCT GAA CTT GCC CG-3’; hCyclinB1-S, 5’-GGT CTG GGT

CGG CCT CTA CCT-3’ and hCyclinB1-AS, 5’-AGC CAG GTG CTG CAT AAC TGG AA-3’; hPLK1-S, 5’-CCT GCA CCT CAG CAA CGG CA-3’ and hPLK1-AS, 5’-CCA TAG TGC GGG CGT AGC GG-3’; hSLUG-S, 5’-GAT GCC GCG CTC CTT CCT GG-3’ and hSLUG-AS, 5’-GGG GGA CTC ACT CGC CCC AA-3’; hE-cadherin-S, 5’-CGG GAA TGC AGT TGA GGA TC-3’ and hE-cadherin-AS, 5’-AGG ATG GTG TAAGCG ATG GC-3’; hVimentin-S, 5’-GAG AAC TTT GCC GTT GAA GC-3’ and hVimentin-AS, 5’-GCT TCC TGT AGG TGG CAA TC-3’; hN-cadherin-S, 5’-GAC GGT TCG CCA TCC AGA C-3’ and hN-cadherin-AS, 5’-TCG ATT GGT TTG ACC ACG G-3’; hSNAIL-S, 5’-ACCC CAA TCG GAA GCC TAA CT-3’ and hSNAIL-AS, 5’-GGTCGTAGGGCTGCTGGAA-3’; hPCNA-S, 5’-ACT AAC TTT TGC ACT GAG GTA CC-3’ and hPCNA-AS, 5’-GTA TTT TAA GTG TCC CAT ATC CGC-3’; hKI-67-S, 5’-TCC TTT G GT GGG CAC CTA AGA CCT G-3’ and hKI-67-AS, 5’-TGA TGG TTG AGG TCG TTC CTT GAT G-3’; hTWIST-S, 5’-GCT TCC TGT AGG TGG CAA TC-3’ and hTWIST-AS, 5’-TCT GGA GGA CCT GGT AGA GG-3’; hOCT4-S, 5’-AAG CGA TCA AGC AGC GAC GAT-3’ and hOCT4-AS, 5’-GGA AAG GGA CCG AGG AGT ACA-3’; hALDH1-S, 5’-GGC TTT ATG TAA ATG GAG GCT GC-3’ and hALDH1-AS, 5’-GTC AAG CTG GGG CTC TTC TT-3’; hGAPDH-S, 5’-GGA GCG AGA TCC CTC CAA AAT-3’ and hGAPDH-AS, 5’-GGC TGT TGT CAT ACT TCT CAT GG-3’. The qPCR was performed in the realplex2 qPCR system (Eppendorf). Relative mRNA levels were normalized to GAPDH.

Protein extraction and Western blotting

Cells were washed with ice-cold PBS and lysed on ice in buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA plus protease inhibitors. Tumor

tissue were homogenized using the TissueLyser (Qiagen) and lysed in buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 10 mM NaF, 20% glycerol, 1% Nonidet P-40 plus protease inhibitors. After centrifugation, protein concentration in supernatants was measured using the BCA protein assay reagent (Thermo Fisher Scientific).

To prepare cytoplasmic and nuclear extract, cell pellets were suspended in CE buffer (10 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, containing protease inhibitor) and incubated on ice for 5 min. An equal amount of CE buffer containing 0.2% NP40 was added to the cell suspension, incubated for 5 min on ice, and centrifuged for 3 min at 6500 rpm at 4 ℃. The supernatant is the cytoplasmic extract. The pellet was resuspended in NE buffer (20 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, containing protease inhibitors) and vortexed at full speed for 1 min. The nuclear extract suspension underwent three cycles of freeze (-80℃, 15 min) and thaw (37 ℃, 1 min). Between each cycle of freeze/thaw the suspension was vortexed for 1 min at full speed. The suspension was then spun at full speed for 15 min at 4 ℃. The supernatant is the nuclear extract.

Lysates were mixed with beta-mercaptoethanol containing sample buffer, heated to 95 ℃ for 10 min. The lysates were separated using SDS-PAGE and transferred onto PVDF membrane for western blotting. The following antibodies and dilutions were used for Western blotting: rabbit anti-FOXM1 (C-20) (1:5000, SantaCruz sc-502, recognizing FOXM1 C-terminus), rabbit anti-FOXM1 (K-19) (1:5000, SantaCruz sc-500, recognizing FOXM1 N-terminus), rabbit anti-CDC25B (1:1000, SantaCruz sc-326), rabbit anti-CyclinB1 (M-20) (1:500, SantaCruz sc-595), goat anti-KI-67 (1:500, SantaCruz sc-7846), rabbit anti-His-probe (G-18) (1:500, SantaCruz sc-804), mouse anti-PCNA (1:10000, abcam ab29), rabbit

anti-E-Cadherin (1:500 abcam ab15148), rabbit anti-Vimentin (1:1000, Sigma HPA001762), rabbit anti-N-Cadherin (1:1000, abcam ab18203), mouse anti-GFP (1:500, Beyotime AG281), mouse anti-SLUG (C-7) (1:500, SantaCruz sc-166902), mouse anti-Lamin A/C (346) (1:1000, SantaCruz sc-7293), goat anti-SMAD3 (1:1000, Abcam ab75512), rabbit anti-PLK1 (1:1000, CUSABIO, CSB-PA004668), mouse anti-B-Myb (1:500, SantaCruz sc-390198), rabbit anti-LIN9 (1:500, Abcam ab214230), rabbit anti-GST (1:1000, Abcam ab19256), mouse anti-β-actin (1:10000, Abcam ab49900), and mouse anti-α-Tubulin (1:1000, SantaCruz sc-53646). The signals from the primary antibody were amplified by horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1:10000, GE LNA934VAE), anti-mouse IgG (1: 10000; BioRad 170–6516), anti-Goat IgG (1:1000, Beyotime A0181), and detected with Super Signal West Femto Maximum Sensitivity Substrate (Thermo) by Kodak 4000 MM Imaging System (Kodak). AVI-tagged proteins were detected by HRP-labeled Streptavidin (1:5000, Beyotime A0303) directly. Membranes were cut horizontally to detect with multiple antibodies. The bands of Western blotting were quantified by ImageJ software.

Electrophoretic mobility shift assays (EMSAs)

The double-strand DNA (dsDNA) probe was synthesized by Sangon (Shanghai) Co., Ltd, China, based on the following sequence: dsDNA probe: forward strand 5'-FAM-TTT GTT TAT TTG TTT GTT TAT TTG-3'(Hot) or 5'-TTT GTT TAT TTG TTT GTT TAT TTG-3'(Cold), and reverse strand 5'-CAA ATA AAC AAA CAA ATA AAC AAA-3'. Proteins (2 μM) were incubated with the FAM-labeled dsDNA probe (50 nM) in binding buffer (20 mM Tris-Cl, 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.2 mM DTT, pH 7.6) for 30 min on ice. The dosages of unlabeled cold probe Apt (5 μM) or M1-138 of increased dosage (0.5, 1,

and 2 μM) for competitive experiment or interaction experiment were added in reactions. The reactions were resolved in 4% native polyacrylamide gel electrophoresis in 0.5XTBE and visualized with Kodak 4000 MM Imaging System (Kodak) (EX: 465 nm, EM: 535 nm for FAM).

Confocal imaging

Cells (1×10^4 cells/well) were seeded in a 35-mm glass bottom petri dish (MatTek) and transfected by Lipofectmin 2000 (Invitrogen) according to the manufacturer's instructions with pCMV-FOXM1-GFP (5 μg) and pCMV-SMAD3-RFP (5 μg). One day later M1-138 (8 μM) was added. FOXM1-GFP or SMAD3-RFP was imaged with a FluoView FV1000 confocal imaging system (Olympus). The fluorescent GFP or RFP was excited by laser at 488 nm or 559 nm respectively.

Luciferase activity assays

293T cells (1×10^5 cells/well in a 12-well plate) were transfected with certain luciferase reporter constructs (1 μg) and expression vectors (1 μg). The pRL-CMV plasmid (20 ng) was used as the loading control for each transfection. The luciferase enzyme activities were measured 48 h later with the Dual-Luciferase Assay System (Promega) following the manufacturer's instructions.

Cell cycle analysis

MDA-MB-231 cells ($2X10^5$) were plated in 6-well plates for 24 h and the medium was replaced with fresh culture medium containing R9-GFP or M1-138 (8 μM). After incubation for 24 h, the cells were harvested by trypsinization and fixed with 4 ℃ 70% ethanol. Intracellular DNA was stained with 50 μg/mL propidium iodide in the dark for 30 min at

room temperature, the cells were filtered and analyzed for DNA content on Quanta SC flow cytometer (Beckman).

Hemolysis assays

Hemolysis assays were performed according to the standard protocols. Briefly, a 10% suspension of erythrocytes (v/v) was prepared by removing from the mouse blood and suspended in normal saline (NS, 0.9%). The suspension (100 μL per sample) were treated with different concentrations of M1-138 (6.5, 12.5, 25, 50, 100, 200 μg/mL) for 3 h at 37 ℃, while sterile double distilled water and PBS were used as the positive and vehicle controls, respectively. After centrifugation at 1200 g for 15 min at 4 ℃, 100 μL supernatant was added into a 96-well plate and the absorbance was measured at 540 nm using a Synergy HTX microplate reader (Biotek). Hemolysis rate % = [(OD sample - OD negative) / (OD positive - OD negative)] × 100%.

Toxicity test

All animal experiments were conducted in accordance with institutional animal care and use guidelines, following approval by the Laboratory Animal Center of Hunan, China (Protocol No. SYXK [Xiang] 2013-0001). ICR/JCL mice (6 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). Mice were randomized into four groups (three mice per group) and injected with different doses of M1-138 (0.4 mg/mouse, 0.8 mg/mouse, 1.2 mg/mouse and 3.6 mg/mouse) by intraperitoneal injection. The corresponding dosage/body weight was calculated to be 20 mg/Kg, 40 mg/Kg, 60 mg/Kg, and 180 mg/Kg. The control group was intraperitoneally injected with PBS. The mice were continuously observed for diet and activity for 14 days.

Immunohistochemistry

The separated tumor tissues were fixed with 4% paraformaldehyde and embedded in paraffin. For immunohistochemistry staining, the tumor sections (4 μm) were dewaxed and rehydrated followed by endogenous peroxidase quenching, antigen retrieval (saline sodium citrate, microwaving), and non-specific binding site blocking. The sections were incubated subsequently with goat anti-KI-67 (1:200, SantaCruz sc-7846), goat anti-CDC25B (1:200, SantaCruz sc-6948), followed by the incubation of a horseradish peroxidase conjugated anti-goat secondary antibody. Color was detected with 3,3'-diaminobenzidine and pictures were taken at X200 magnification using a TE2000 microscope (Nikon).

References

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