Supplementary material and method

Cell culture

Hepatocellular carcinoma cell lines (HepG2, SMMC-7721, MHCC-97H) were purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences (China). HEK 293T cells were purchased from American Type Culture Collection (USA). The HepG2 cells and HEK 293T cells were cultivated in DMEM media (Invitrogen, Carlsbad, CA), and SMMC-7721 and MHCC-97H cells were cultivated in RMPI-1640 media (Invitrogen, Carlsbad, CA). All the media were supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, USA). Cells were cultured at 37°C in a humidified incubator containing 5% CO2.

Lentiviral infection

HEK 293T cells were seeded in fresh DMEM 24 h before transfection. Cells were co-transfected with destination plasmids and package plasmids (psPAX2 and pMD2G) using Lipofectamine^{TM} 2000 transfection reagent (Invitrogen, Carlsbad, CA). Media containing viral particles was harvested at 48 h and 72 h after transfection. SMMC-7721 cells were incubated with virus-containing media and culture media at a ratio of 1:1 for 24 h in the presence of 8 μg/mL polybrene (Sigma Aldrich, St. Louis, USA), followed by ten days of selection with antibiotics.

Wound healing assay

Cells were seeded in six-well plates and incubated overnight until they reached to 80%

confluency. The white pipette tips were used to introduce a scratch in the monolayers. The cells were washed with PBS twice to remove the floating cells. The cells were then incubated in the serum-free media with OA (50 μM) or DMSO in the presence of TGF-β1 for 48 h. The scratch healing was determined by measuring the shortest distance between scratch edges in each field of view. Five random fields were analyzed from each well.

Cell Invasion Assay

The transwell chambers (Merck & Millipore, USA) were coated with Matrigel (Corning Costar, USA) at 37°C for 1 h. Cells (1×10^5 cells/well) were plated in the upper chamber with serum-free media. The media containing 10% fetal bovine serum and OA (50 μM) or DMSO was poured into the lower chamber. After 24 h, cells were incubated with TGF-β1 for another 24 h. Cells in the upper compartment were then removed. The migrated cells were fixed with 4% paraformaldehyde, stained with crystal violet, and manual counted. Five random fields were analyzed from each well.

Apoptosis assay

Cells were treated with of OA (0, 12.5, 25, 50 μM) for 48 h. After incubation, cells were stained with Annexin V-FITC and PI for 10 min and analyzed using flow cytometry according to the protocol of Annexin V-FITC apoptosis detection kit (Vazyme, Nanjing, China).

Cell viability assay

Cell viability was measured using the CCK-8 assay (Vazyme Biotec, Nanjing, China). 4×10^3 cells were treated with OA for 48 h. Absorbance (A) of the final formazan was measured spectrophotometrically at 450 nm by Multiskan™ FC microplate photometer (Invitrogen, Carlsbad, CA). The inhibition ratio (%) was calculated as follows: $((A_{control}-A_{treated})/A_{control}) \times 100\%$. $A_{treated}$ and $A_{control}$ are the average absorbance values of three parallel experiments from the treated and control groups.

Extraction of cytoplasm/nucleus proteins

Cells were incubated with OA (0, 12.5, 25, 50 μM) for 24 h. Nuclear and cytosolic protein extracts were prepared using a nuclear/cytosol fractionation kit (Invitrogen, Carlsbad, CA). Briefly, cells were collected in a tube, and an appropriate volume of ice-cold CERI solution was added. The solution containing tube was vortexed and kept on ice for 10 min. Ice-cold CERII solution was added to the tube, and the tube was vortexed and kept on ice for 1 min. Then, centrifuge at 16000 g for 5 min. The supernatant (cytoplasmic extract) was transferred to to a new tube. The pellet fraction was suspended with an appropriate volume of ice-cold NER solution. The solution was vortexed and kept on ice for 40 min. Followed by centrifugation at 16000 g for 10 min. The supernatant (nuclear extract) was transferred to a new tube. The extracts were stored at -80°C until use.

Western blot

Cells were lysed in RIPA lysis buffer (Thermo Fisher, USA) supplemented with protease inhibitor cocktail (Thermo Fisher, USA). The total cell lysates were subjected to immunoblot assay using primary antibodies, followed by incubation with appropriate HRP-conjugated secondary antibodies. Blots were detected by chemiluminescence (Thermo Fisher, USA). The following primary antibodies were used: (1) GAPDH, 1:10000; (2) β-Actin, 1:5000; (3) Smad2/3, 1:2000; (4) pSmad2/3, 1:1000; (5) E-cadherin, 1:1000; (6) N-cadherin, 1:1000; (7) Twist1, 1:2000; (8) Vimentin, 1:500; (9) C/EBPβ, 1:1000; (10) pC/EBPβ, 1:1000; (11)Acetyl-lysine, 1:1000; (12) c-Myc, 1:2000; (13) HDAC1, 1:1000; (14) GFP, 1:5000; (15) NAG-1, 1:2000; (16) p300, 1:1000; (17) Lamin A/C, 1:1000; (18) α/β-Tubulin, 1:1000.

Immunoprecipitation

The cells were lysed in IP buffer [50 mM tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% NP-40 (pH 7.4)] containing protease inhibitor cocktail (Thermo Fisher, USA). The cell lysate was sonicated, clarified, and incubated with antibodies against control immunoglobulin G, C/EBPβ (1:100) overnight at 4°C, followed by incubation with protein A/G agarose beads (Santa Cruz Biotechnology, USA) at 4°C for 4 h. The immunocomplexes were subjected to immunoblot analysis.

Dual-luciferase reporter assay

For luciferase assay, plasmids and pRL-TK (used as a transfection efficiency control) were transfected using Lipofectamine™ 2000 transfection reagent and the luciferase

activity was detected with dual luciferase reporter assay kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's protocol. The luciferase activity was calculated as a ratio of the firefly luciferase signal to the renilla luciferase signal.

Immunofluorescence

The treated cells were collected and seeded onto coverslips for immunofluorescence. The glass coverslips were washed twice with cold PBS for 5 min, fixed with 4% paraformaldehyde for 20 min and incubated with 0.2% Triton X-100 (Sigma Aldrich, St. Louis, USA) for 20 min. After incubation, the cells were blocked with PBS containing 3% BSA (Yisheng, Shanghai, China) for 1 h at room temperature and incubated with primary anti-Smad2/3 (1:500), Alexa Fluor^{TM} 488 phalloidin, C/EBPβ (1:200) at 4°C overnight. Following the PBS wash, cells were incubated with anti-rabbit Alexa Fluor 488 secondary antibody (1:200; Invitrogen, Carlsbad, CA) for 1 h at 37°C and stained with DAPI (Invitrogen, Carlsbad, CA). Fluorescence was observed with a laser confocal microscope (Zeiss LSM 800, Germany).

Protein expression and purification

The recombinant GST-HDAC1 fusion proteins were expressed in *E.coli* BL21 cells and purified with Pierce™ glutathione agarose (Thermo Fisher, USA). Cells were lysed in STE buffer (20 mM Tris–HCl, 137 mM NaCl, 1 mM PMSF, 0.1% Tween 20, and 5 mM DTT, pH 7.6) and eluted with elute buffer (30 mM L-glutathione reduced, 200 mM Tris-HCl, pH 8.0).

Molecular Docking

The crystal structures of HDAC1 (PDB ID: 5ICN) were downloaded from the Protein Data Bank (PDB) and prepared by the Protein Preparation Wizard in the Schrodinger suite. The small molecules were prepared using the LigPrep module of Schrodinger. The Glide module (extra precision [XP] mode) was used for molecular docking due to its excellent performance through a self-docking analysis. The ten best poses of each ligand were minimized by a post docking program, and the best pose was saved for further analysis.

Microscale thermophoresis

Monolith NT.115 (Nanotemper technologies, Munich, Germany) was used for microscale thermophoresis (MST) assays. Recombinant WT or mutant HDAC1 proteins were labeled with FITC (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. 20 nM of labeled WT or mutant HDAC1 were incubated with OA at indicated concentrations in PBS for 5 min at room temperature. Thermophoresis was then observed at 25°C with 2-10% laser power and medium LED power.

GST pull-down assay

Purified GST-fusion protein was incubated with Biotin-OA (10 μM) at 4°C overnight. The next day, 20 μL soft release avidin resin (Promega, Madison, Wisconsin, USA) was added to the solution, and the solution was rotated for 2 h at 4°C. The resins were

washed in PBS buffer three times and centrifuged. The resins were then resuspended in a 24 μL SDS-sample buffer (0.5 M Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol, 4‰ bromophenol blue) and boiled for 10 min. The samples were then subjected to immunoblot analysis.

Lung metastasis mice model

Five-week-old female BALB/c nude mice were injected with SMMC-7721 cells (2×10^6 cells/mouse) intravenously. After one week, the mice were treated with saline or OA (200 mg/kg) via intragastric administration every other day for six weeks. The bodyweight of the mice was measured every three days. The survival rate was determined by observation when the animals became moribund.

The animals were housed under standard specific-pathogen-free conditions. The animal studies were carried out according to protocols approved by the Animal Welfare and Ethics Committee of China Pharmaceutical University.

Hematoxylin and eosin (H&E) staining

Tissues were fixed in 4% PFA and embedded in paraffin. The embedded tissues were cut into at 5 μm sections, deparaffinized and stained with hematoxylin and eosin (H&E).

Supplementary Table 1: List of primers used in this study					
Vector	**Gene**		**Primer sequence**	**PCR product (bp)**	**Restriction enzyme**
pLenti-GFP vector	HDAC1（NM_004964.3）	Forward	CTGGGTCGGACGCTGAG	1966	
		Reverse	GCCAGTGGGAAGGTACGAAA		
		Forward	GCTTCGAATTCTGCAGTCGACGCGCAGACGCAGGGCACC	1490	SalI and Asc I cut into 5549, 5592bp
		Reverse	CAAGAAAGCTGGGTCGGCGCGCCTCAGGCCAACTTGACCTCCTCCTTG		
	HDAC1-G149A	Forward	gcttcgaattctgcagtcgacGGCGCAGACGCAGGGCAC	1490	SalI and Asc I cut into 5549, 5592bp
		Reverse	caagaaagctgggtcggcgcgccTCAGGCCAACTTGACCTCCTC		
pGEX-6P-1	HDAC1	Forward	CCCCTGGGATCCCCGGAATTCGCGCAGACGCAGGGCACC	2558	EcoR I and Not I cut into 954, 975bp
		Reverse	TCAGTCAGTCACGATGCGGCCGCTCAGGCCAACTTGACCTCCTC		
	HDAC1-N95A	Forward	AGAGATTCgcgGTTGGTGAGGACTGTCCAGTATTCG	6416	
		Reverse	ACCAACcgcGAATCTCTGCATCTGCTTGCTGT		
	HDAC1-D99A	Forward	TGAGGcgTGTCCAGTATTCGATGGCCTGTTTGA	6416	
		Reverse	AATACTGGACAcgCCTCACCAACGTTGAATCTCTGC		
	HDAC1-G149A	Forward	GGCATCTGcgTTCTGTTACGTCAATGATATCGTCTTG	6416	
		Reverse	TAACAGAAcgCAGATGCCTCGGACTTCTTTGCA		
	HDAC1-F205A	Forward	AGTATGGAGAGTACgcgCCAGGAACTGGGGACCTACG	6416	
		Reverse	cgcGTACTCTCCATACTTATGAAAGGACACAG		
pLKO.1 hygro vector	shNAG-1 1	Forward	CCGGCCGGATACTCACGCCAGAAGTCTCGAGACTTCTGGCGTGAGTATCCGGTTTTTG		Age I and EcoR I cut into 2366, 2390bp
		Reverse	AATTCAAAAACCGGATACTCACGCCAGAAGTCTCGAGACTTCTGGCGTGAGTATCCGG		
	shNAG-1 2	Forward	CCGGACGCTACGAGGACCTGCTAACCTCGAGGTTAGCAGGTCCTCGTAGCGTTTTTTG		Age I and EcoR I cut into 2366, 2390bp
		Reverse	AATTCAAAAAACGCTACGAGGACCTGCTAACCTCGAGGTTAGCAGGTCCTCGTAGCGT		

Supplementary Table 2				
Primers for qPCR				
Gene	**NM number**		**Primer sequence**	**qPCR product (bp)**
NAG-1	NM_001923.5	Forward	CAGTCGGACCAACTGCTGGCA	361
		Reverse	TGAGCACCATGGGATTGTAGC	
PAI-1	NM_000602.5	Forward	CACGAGTCTTTCAGACCAAGAG	616
		Reverse	CACACAAAAGCTCCTGTAAGC	
MMP2	NM_004530.6	Forward	TTCCCCTTCTTGTTCAATGG	376
		Reverse	ATTTGTTGCCCAGGAAAGTG	
MMP9	NM_004994.3	Forward	GCTCTTCCCTGGAGACCTG	392
		Reverse	ACACGCGAGTGAAGGTGAG	
CDH1	NM_001317185.2	Forward	GGCTCAAGCTATCCTTGCAC	314
		Reverse	CTTGAGCCCCAGAGTTTGAG	
TWIST1	NM_004628.5	Forward	GGCTCAAGCTATCCTTGCAC	156
		Reverse	GGCTCAAGCTATCCTTGCAC	
HDAC1	NM_004964.3	Forward	CGCCCTCACAAAGCCAATG	108
		Reverse	CTGCTTGCTGTACTCCGACA	
GAPDH	NM_001357943.2	Forward	TCTGGTAAAGTGGATATTGTTG	102
		Reverse	GATGGTGATGGGATTTCC	

Supplementary Table 2			
Primers for CHIP			
Gene		**Primer sequence**	**qPCR product (bp)**
CEBPB	Forward	GCCCTAGCTTTACTAGGAGCG	252
	Reverse	CTGAGAGCCATTCACCGTCC	
GAPDH	Forward	TACTAGCGGTTTTACGGGCG	166
	Reverse	TCGAACAGGAGGAGCAGAGAGCGA	

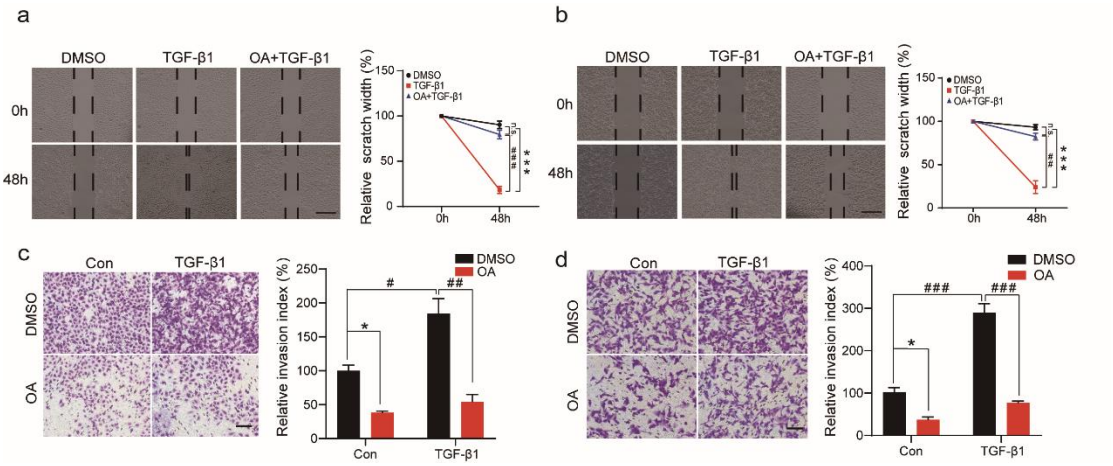


Figure S1. Oroxylin A inhibits TGF-β1-induced migration of HCC cells

(a and b) Wound healing assay. MHCC-97H cells and HepG2 were treated with 50 μM OA or DMSO followed by co-incubating with TGF-β1 (10 ng/mL) for a total of 48 hours. Representative images photographed at 0 h and 48 h. Scale bar: 100 μm. And the quantification of the wound healing. (c and d) After MHCC-97H cells and HepG2 cells were pretreated with OA (50 μM) or DMSO in the presence or absence of TGF-β1 (10 ng/mL) for a total of 48 h and then a Matrigel-based transwell assay was used to evaluate their invasive properties. Scale bar: 50 μm. And the quantification of the cell invasion rates. Data are presented as mean ± SD.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with normal group or referred group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with TGF-β1 group. n.s. not significant. Differences are tested using two-way ANOVA.

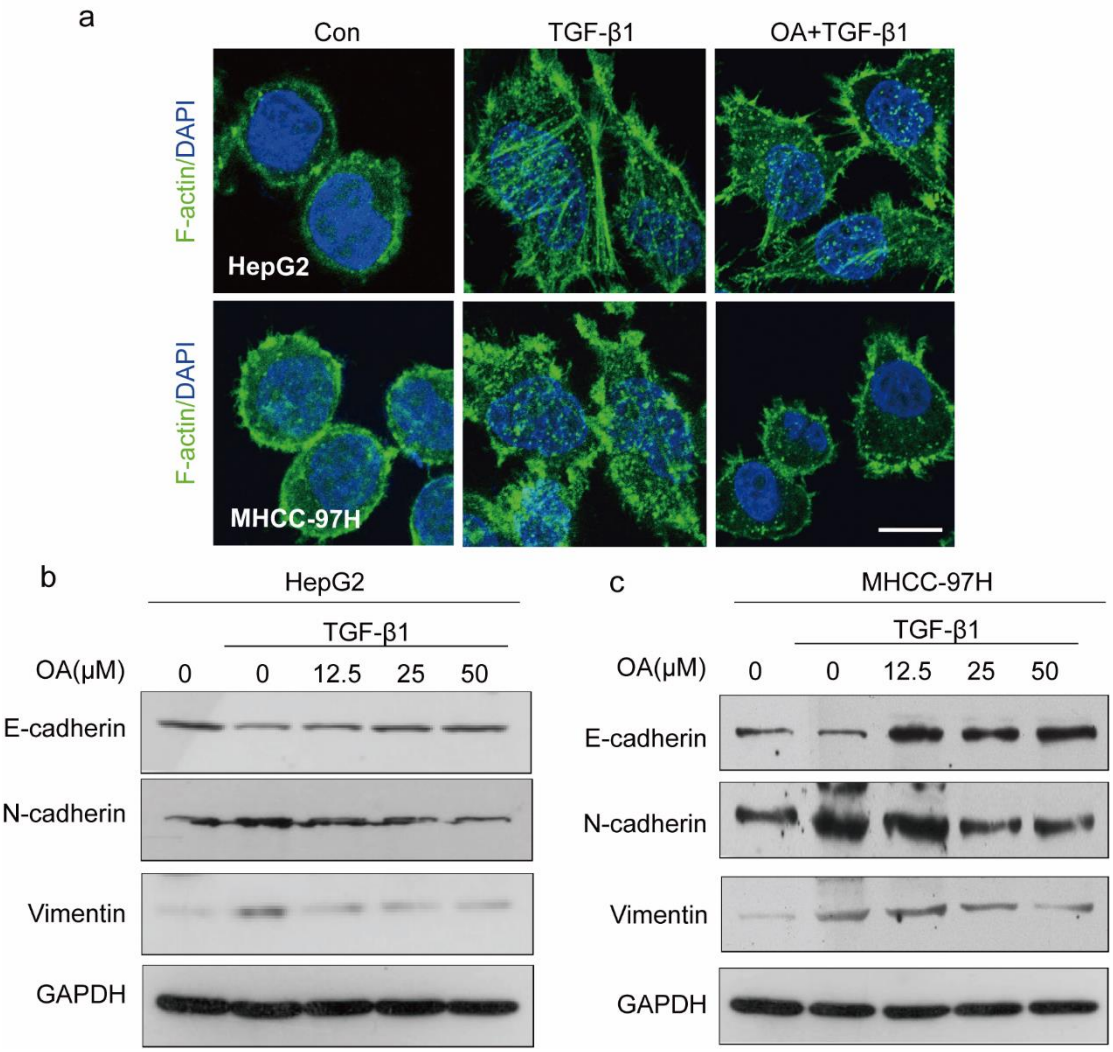


Figure S2. Oroxylin A represses EMT process of HCC cell

(a) MHCC-97H cells and HepG2 cells were treated with OA (50 μM) or DMSO in the presence of TGF-β1 (10 ng/mL) for 48 h. Immunofluorescence assay was performed to detect the structure of F-actin with phalloidin staining. Scale bar: 5 μm. (b and c) MHCC-97H cells and HepG2 cells were treated with OA as indicated doses in the presence or absence of TGF-β1 (10 ng/mL) for 48 h, and western blot assay was used to evaluate expression level of E-cadherin, N-cadherin and Vimentin.

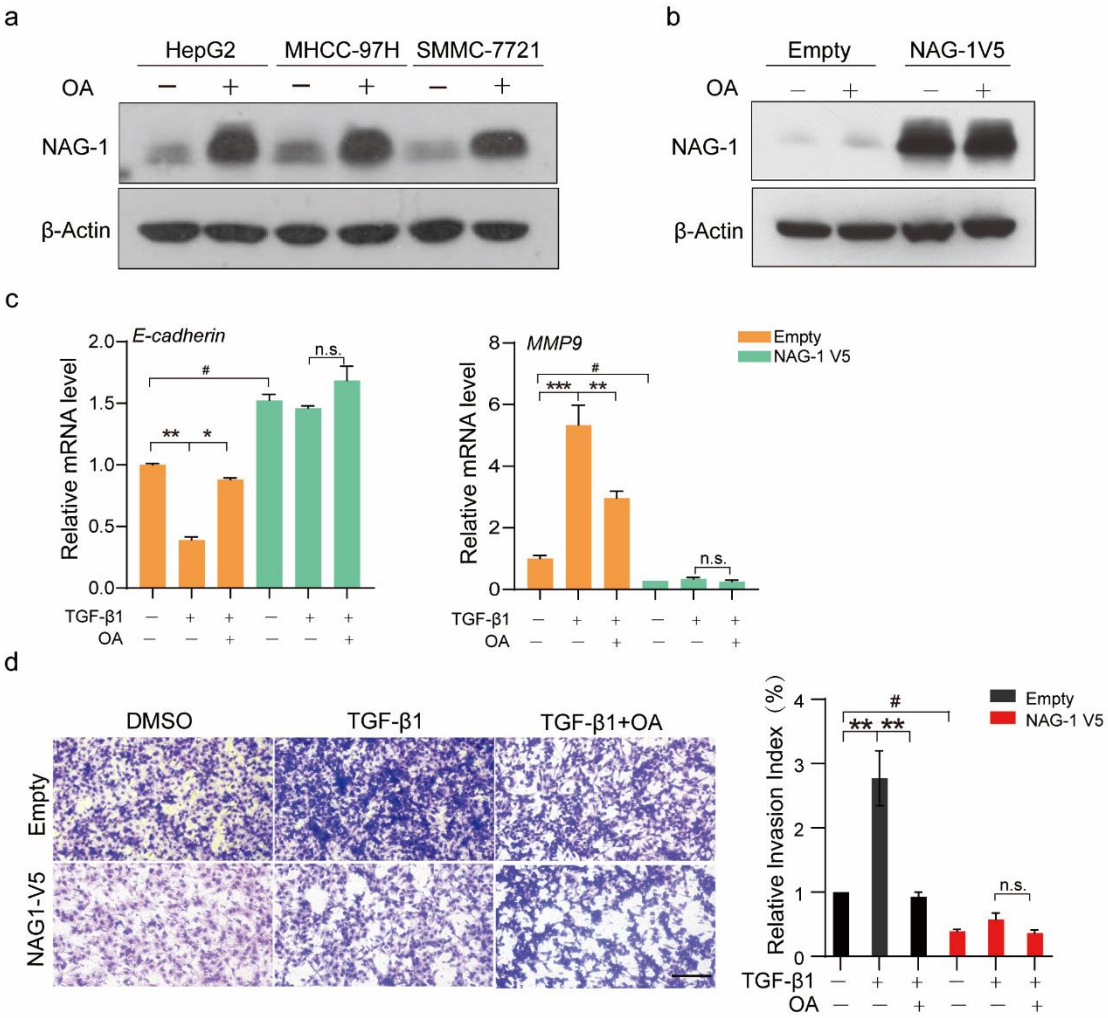


Figure S3. Upregulation of NAG-1 attenuated HCC cells metastasis.

(a) SMMC-7721 cells, MHCC-97H cells and HepG2 cells were treated with OA (50 μM), and the protein level of NAG-1 was evaluated by western blot and β-Actin was used as a loading control. (b) SMMC-7721 cells were transiently transfected with pcDNA3.1 NAG-1 V5 or empty construct for 24 h. The efficiency of NAG-1 overexpression was evaluated by western blot. (c) qPCR analysis of *E-cadherin* and *MMP9* mRNA levels in SMMC-7721 cells transiently transfected with pcDNA3.1 NAG-1 V5 or empty construct as indicated treatment. (d) SMMC-7721 cells were treated with OA (50 μM) or DMSO in the presence or absence of TGF-β1 (10 ng/mL) for 48 h. The invasive ability was evaluated by transwell invasion assay. Scale bar:

50 μm. Data are represented as mean ± SD. for three independent experiments. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$ compared with normal group or referred group. $^{\#}P <$ 0.05 compared with NAG-1 V5 group. n.s., not significant. Differences are tested using two-way ANOVA.

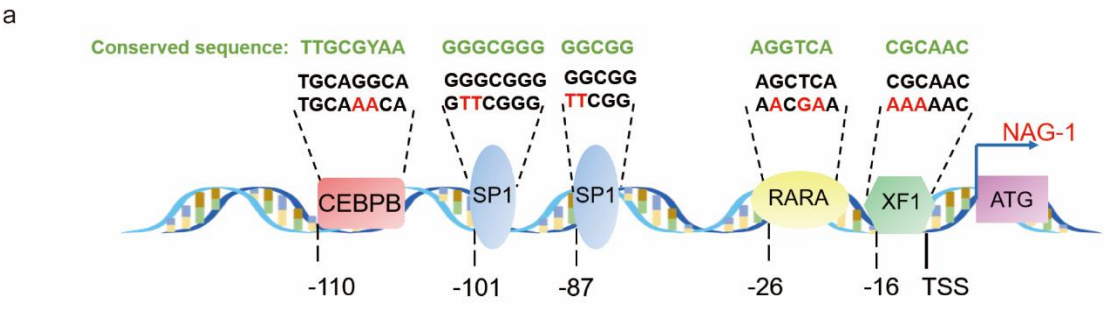


Figure S4. Oroxylin A activated NAG-1 transcription through C/EBPβ binding motif

(a) Schematic representation of the mutation of transcription factor-binding sites on NAG-1 promoter (pNAG-1-133/+41)

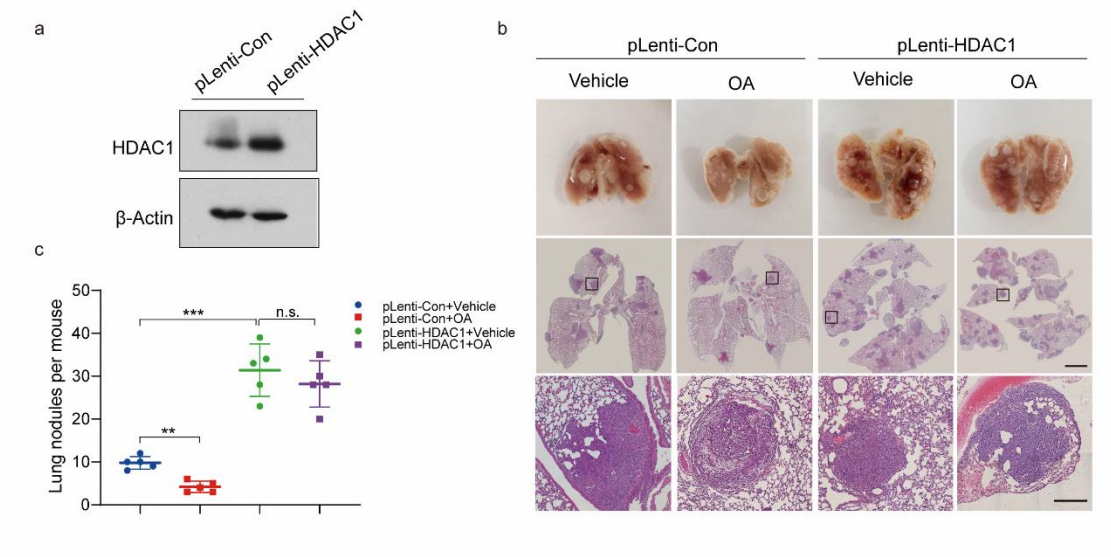


Figure S5. Upregulated HDAC1 attenuated anti-metastasis effect of OA *in vivo*.

(a) Western blot assay of HDAC1 and β-Actin in SMMC-7721 cells stably transfected with pLenti-Con and pLenti-HDAC1. (b) BABL/c nude mice (n=5) were intravenously inoculated with SMMC-7721 cells stably expressing pLenti-Con or pLenti-HDAC1. Representative photographs and H&E staining of lung sections Scale bar =4 mm. High magnification pictures (200X) of H&E staining. Scale bar =200 μm. (c) Quantification of nodules in lungs from indicated mice (n=5). Data are represented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.005$, n.s., not significant. Differences are tested using Student's *t*-test.

Fig 2-c

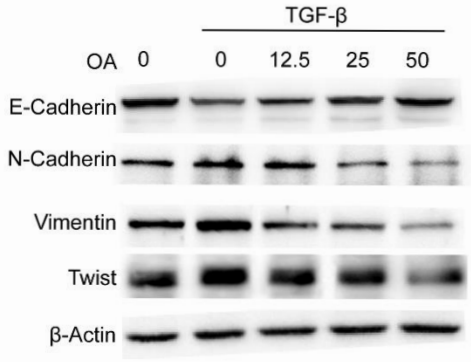


Fig S2-b

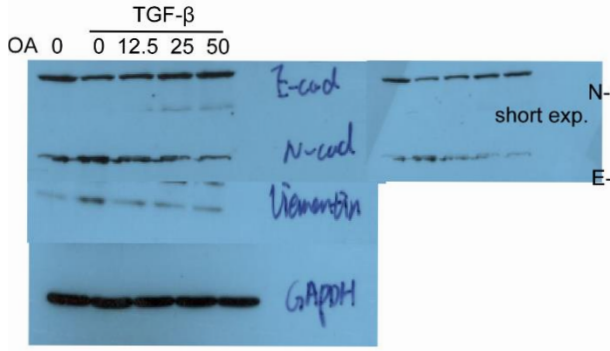


Fig S2-c

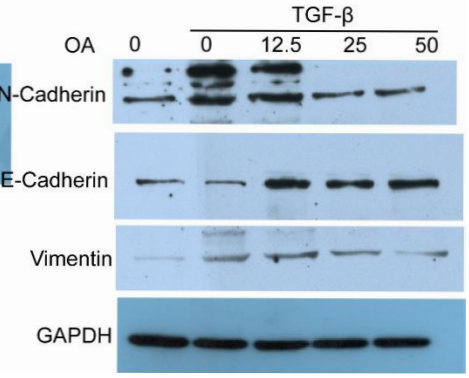


Fig3.d

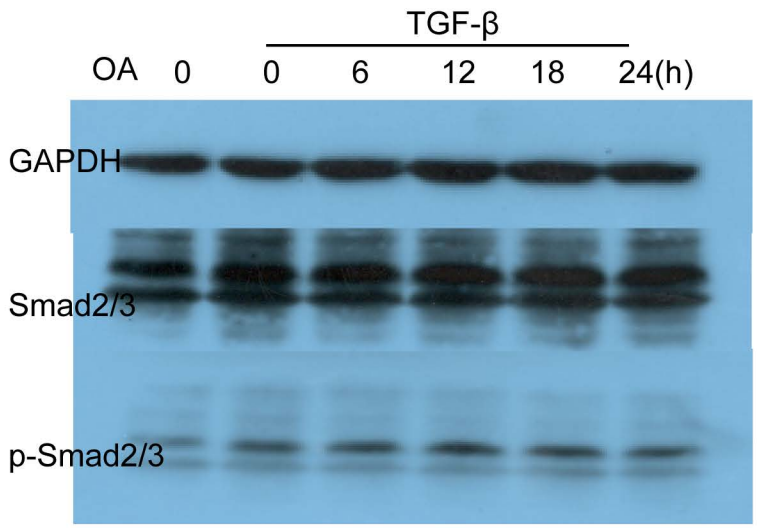


Fig3.e

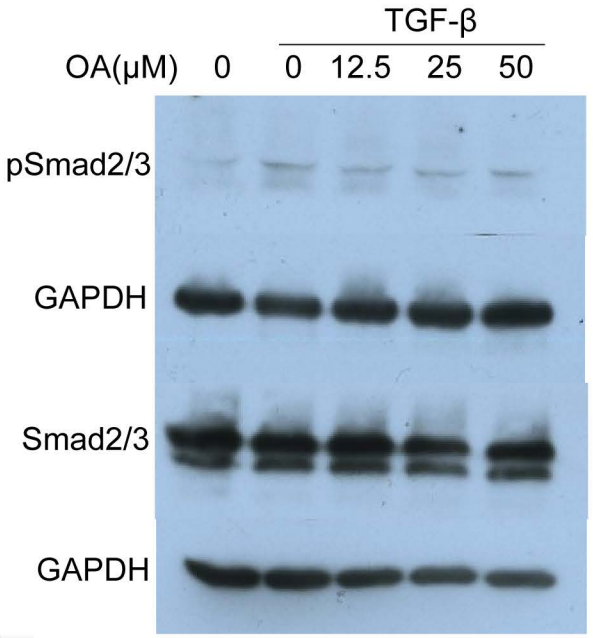


Fig4.a

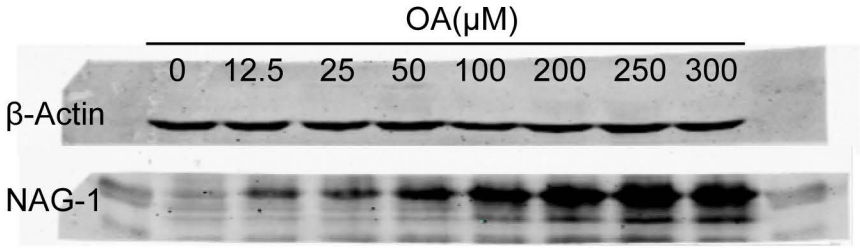
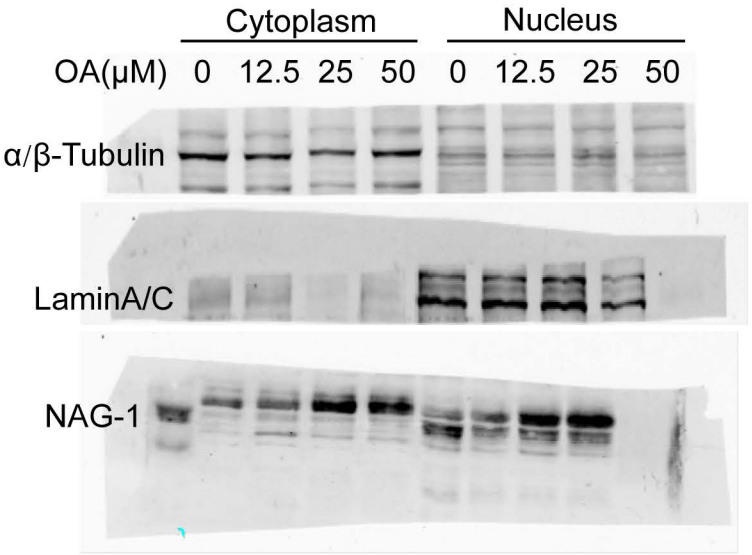


Fig4.b



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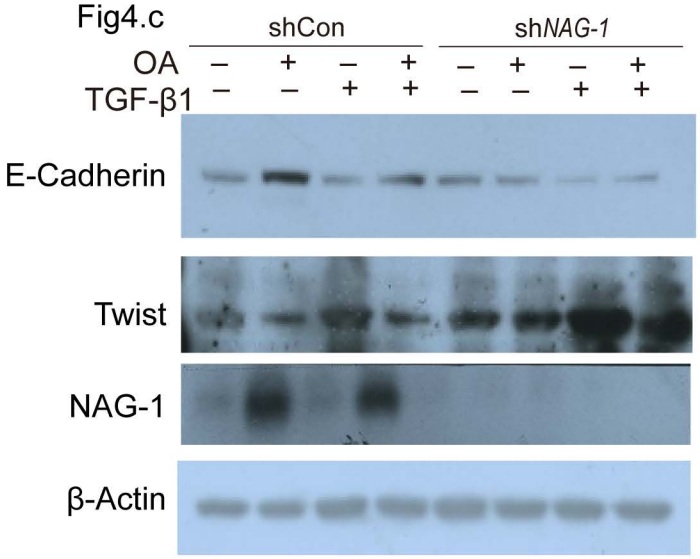
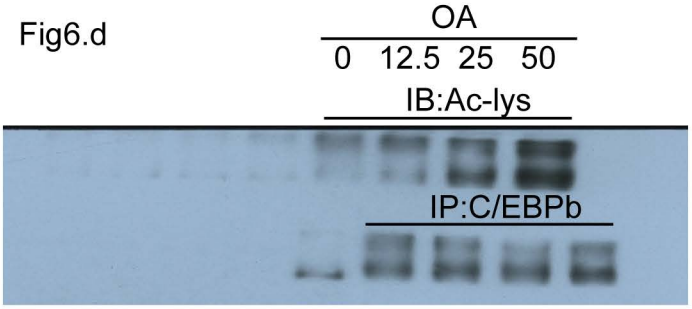


Fig6.d



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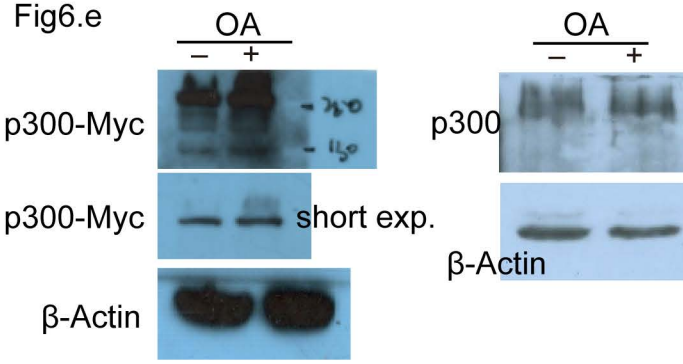


Fig6.f

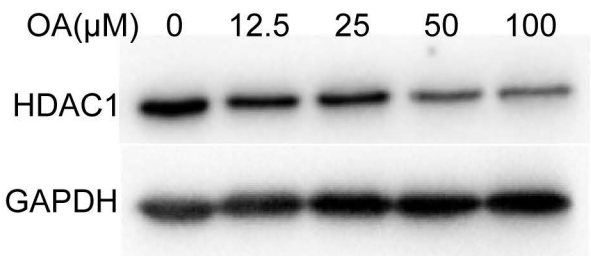


Fig6.i

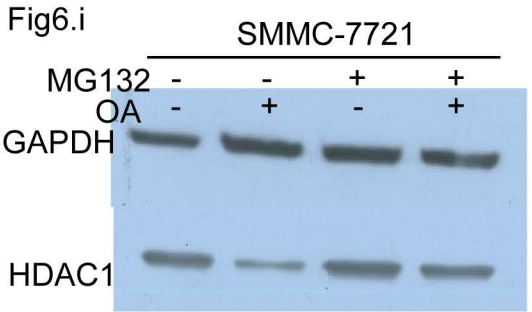


Fig6.h

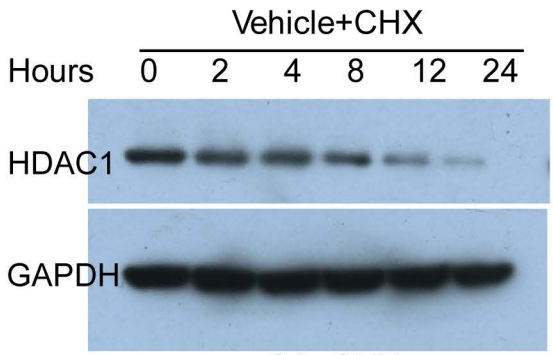


Fig7.c

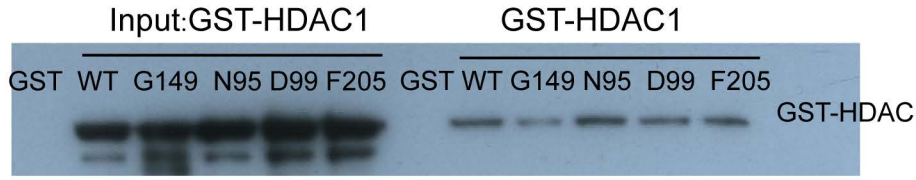
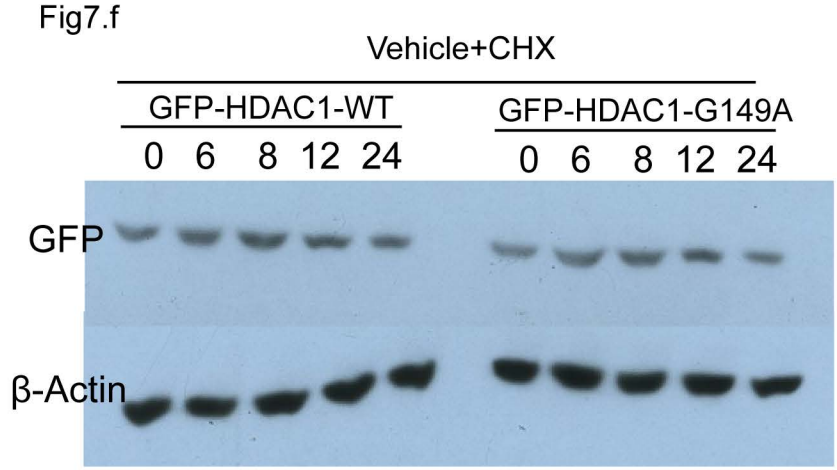


Fig7.f



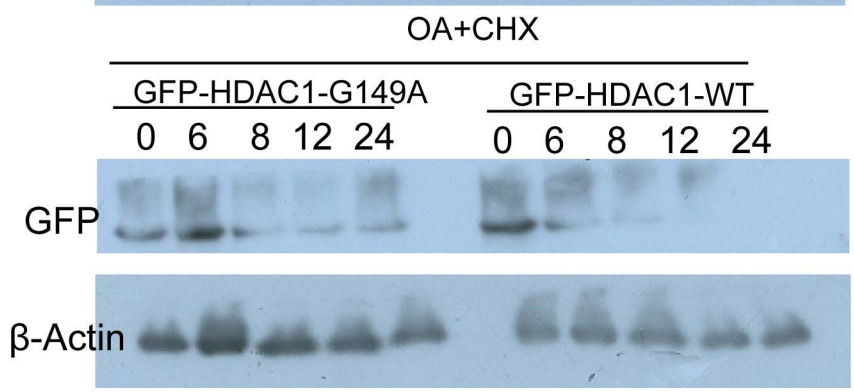
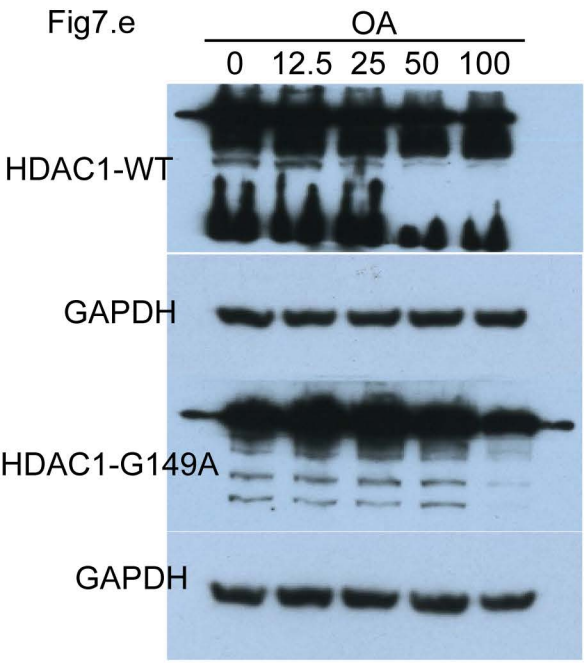
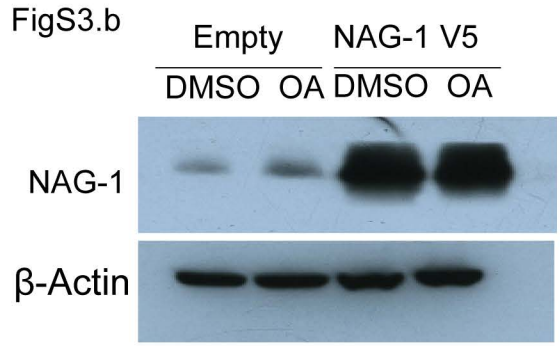


Fig7.e



FigS3.b



FigS3.a

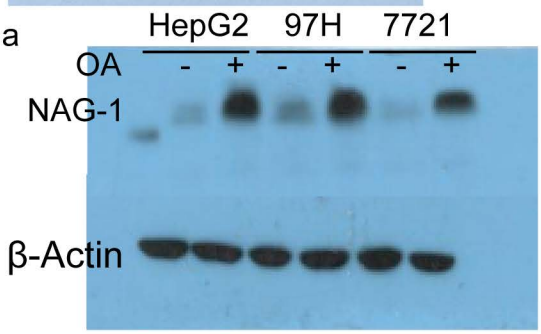
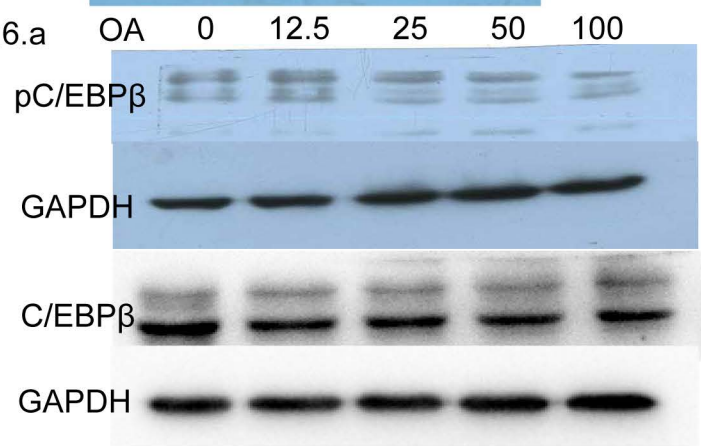


Fig6.a



FigS5.a