Supplementary Materials and Methods

Reagents.

The reagents used were as follows; anti-HDAC2, anti-Erk2, anti-NF-κB1 (p50), anti-RelA (p65), anti-eIF-4E, anti-α-Tubulin, anti-GAPDH antibodies from Santa Cruz Biotechnology, Inc.; anti-p-AKT (Ser473), anti-AKT, anti-p-Erk (Thr202/Tyr204), anti-p-mTOR (Ser2448), anti-mTOR, anti-c-Myc, anti-Snail, anti-Slug, anti-E-cadherin antibodies and Rapamycin from Cell signaling Technology, Inc.; anti-Mdm2 antibody from Millipore; anti-FLAG antibody and recombinant human EGF from Sigma-Aldrich; and horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulins from Amersham Biosciences.

Immunohistochemistry (IHC) of liver tissue

Immunohistochemical staining of p-mTOR, mTOR, and HDAC2 in liver tissue slides were performed using TSA biotin kits according to the manufacturer's instruction (Perkin Elmer). Briefly, slides were deparaffinized and hydrated through graded ethanol to deionized water prior to immunostaining. Slides were incubated with each antibody at 4°C overnight, and with biotin-labeled secondary antibodies for 45 min at 37°C. Amplified signals were detected with DAB chromogen system (DAKO, Glostrup, Denmark) and captured with Axioimager M1 microscope (Carl Zeiss, Oberkochen Germany).

Immunoblotting.

Cells were lysed in protein extraction buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM Na₂P₂O₇, 1 mM Na₃VO₄, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mM PMSF, and protease inhibitor cocktail). Protein concentrations in supernatant were measured using BCA detection reagents, according to the manufacturer's instructions (Pierce, Rockford, IL). SDS-PAGE and immunoblotting were subsequently performed, blots were treated with ECL reagent and chemiluminescence was detected (GE Healthcare).
**Immunofluorescence and confocal microscopy**

Cells were plated and 4-well chamber and then serum-starved overnight. After EGF treatment for 1 hour, cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature, rinsed with PBS, and permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 4% bovine serum albumin in PBS, cells were incubated overnight at 4°C with anti-NF-κBp50, -NF-κBp65, and -HDAC2 (1:100). Then, cells were treated with Alexa Fluor 488- or Texas red X-conjugated secondary antibodies (1:1000, Invitrogen) for 1 hour at room temperature. Nuclei were stained with 10 μg/ml Hoechst 33342 (1:3000) and cells were mounted using Gel/Mount solution (Biomeda, Torino, Italy). Confocal fluorescent images were obtained by a Zeiss LSM510 Meta laser scanning microscope. After sequential excitation (488 nm for Alexa Fluor, and 595 nm for Texas red X), green and red fluorescent images of the same cell were analyzed by ZEN2009 software.

**Cell migration assay.**

Cell migration was measured using a modified Boyden chamber assay (BD Biosciences). Cells were plated on the top surfaces of transwell inserts (8-μm pore size) coated with 1% gelatin, then the inserts were transferred to a 24-well plate. Bottom wells contained serum-free medium containing EGF (50 or 100 ng/mL) or 5% FBS as a chemoattractant. After 24 hours of incubation, cells remaining on the upper surfaces of the inserts were removed with cotton swabs, and cells on the lower surface of the inserts were fixed with 4% formaldehyde and stained with 0.5% crystal violet. The membranes were then mounted onto microscope slides and migrating cells were counted in five different areas under a light microscope.

**Wound healing assay.**

Cells were grown to confluence in culture dishes and transfected with HDAC2-shRNA expression or control vector (pSilencerTM3.1-H1 neo; Ambion, Austin, TX, USA). After overnight starvation in serum-free medium, cell monolayers were scraped with a sterile micropipette tip. Initial gap widths (0
h) and residual gap widths 24 h after wounding were determined using photomicrograph.

**qRT-PCR.**

Total RNA was isolated using TRIzol (Invitrogen), according to the manufacturer’s instructions. cDNA was generated using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA). Relative levels of specific mRNAs were determined using a SYBR Green chemistry system. All PCRs were performed using the iQ™5 Real-Time PCR Detection System (Bio-Rad Laboratories, Philadelphia, PA, US) according to the manufacturer’s instructions. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

**Gene set enrichment analysis.**

Gene Set Enrichment Analysis (GSEA) was performed using a series of gene sets from four independent sources; gene expression differences resulting from three independent HDAC2 knock-down experiments reported in our previous studies (1-3) and a HCC cohort study; (4) all are available in the GEO database. Given a dataset in which genes were rank-ordered by the correlation of their expression levels by phenotype of interest, the basic GSEA test provides a score that quantifies the degree of enrichment of a given gene set at the top (positive correlated) or bottom (negative correlated) of the rank-ordered dataset. The proximities of gene sets were measured using Kolmogorov-Smirnoff (KS) scores (higher score correspond to greater proximity). Observed KS scores were compared to the distribution of 1000 permuted KS scores for all gene sets to assess significance.

**Molecular concept map.**

ConceptGen, an open-source gene set enrichment testing and concept mapping tool, was used to create a network graph to visualize interconnectivity among sets of genes (concepts) (http://conceptgen.ncibi.org/core/conceptGen/index.jsp). To provide the functional relevance of a HDAC2-specific gene set in HCC, we first compared a gene set identified by HDAC2 knock-down in
Hep3B cells (GSE32070) with the HDAC2-correlated gene set identified in a large HCC cohort study (GSE16757) by assessing their molecular correlations using the ConceptGen Molecular Concept Map (MCM), which contains over 20,000 molecular concepts comprising 14 biological knowledge types, for enrichment by disproportionate overlap using a modified Fisher’s extract test.(5) MCM analysis of 1,277 HDAC2-specific genes (1.5-fold up- or down-regulated in HDAC2 knock-down Hep3B cells) with other cancer-related gene sets was performed and an enrichment network was generated.

**Animal tumor models.**

Mouse xenografting was performed as described in our previous study.(1) Briefly, five-week-old male SCID mice (ORIENT BIO Inc. Korea) were used to examine tumorigenicity. To evaluate the effect of HDAC2 knock-down on tumor formation, Hep3B stably over-expressing HDAC2 shRNA or vector control cells were propagated and 5×10⁶ cells were inoculated subcutaneously into dorsal mouse flanks (one flank/mouse; n=5 for shHDAC2, n=5 for shScr). Xenografts were excised, and total protein was extracted for immunoblotting. To induce HCC in mouse and rat liver, diethylnitrosamine was used as previously described. (6, 7)
References


