Supplementary information, Data S1 Materials and Methods

Preparation of H1299 cells extraction for LC-MS

H1299 cells were sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in the lysis buffer (8 M urea, 2 mM EDTA, 3 μM TSA , 50 mM NAM, 10 mM DTT and 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 20,000 g at 4°C for 15 min. Next, the proteins were precipitated with cold 15% TCA for 2 h at -20°C. After centrifugation at 4°C for 15 min, the remaining precipitate was washed with cold acetone for three times. Finally, the proteins were redissolved in buffer (8 M urea, 100 mM NH_4CO_3, pH 8.0).

Trypsin Digestion

Reduction and alkylation reactions were performed before trypsin digestion. Firstly, the protein solution was reduced with 10 mM DTT for 1 h at 37°C and alkylated with 20 mM IAA for 45 min at room temperature. Next, the proteins were diluted by adding 100 mM NH_4CO_3 to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

HPLC Fractionation

The samples were then fractionated by high pH reverse-phase HPLC using Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Firstly, peptides were separated with a gradient of 2% to 60% acetonitrile in 10 mM ammonium bicarbonate (pH 10) over 80 min. Next, the peptides were combined into 10 fractions and dried by vacuum centrifuging.

Affinity enrichment of crotonylated peptides

To enrich lysine crotonylated peptides, tryptic peptides were dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0), and incubated with pre-washed antibody beads (PTM Biolabs) at 4°C overnight with gentle shaking. Then, the beads were washed four times with NETN buffer and twice with ddH_2O. The

bound peptides were eluted from the beads with 0.1% TFA. The eluted fractions were combined and vacuum-dried. Finally, the eluted peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions, followed by LC-MS/MS analysis.

LC-MS/MS Analysis

The enriched crotonylated peptides were dissolved in 0.1% FA, followed with directly loading onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Then, the peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). The gradient was comprised of an increase from 6% to 22% solvent B (0.1% FA in 98% ACN) for 24 min, 22% to 35% for 8 min and climbing to 80% in 5 min then holding at 80% for the last 3 min, all at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system, the resulting peptides were analyzed by Q ExactiveTM Plus hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM plus (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 30; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 5E3 in the MS survey scan with 15.0s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the orbitrap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800. Fixed first mass was set as 100 m/z.

Database Search

The resulting MS/MS data was processed using MaxQuant with integrated Andromeda search engine (v.1.4.1.2). Then, the tandem mass spectra were searched against Swissprot_human database concatenated with reverse decoy database. Trypsin/P was

specified as cleavage enzyme allowing up to 4 missing cleavages, 5 modifications per peptide and 5 charges. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on cysteine was specified as fixed modification and oxidation on methionine, crotonylation on lysine and acetylation on protein N-terminal were specified as variable modifications. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 1%. Minimum peptide length was set at 7. All the other parameters in MaxQuant were set to default values. The site localization probability was set as > 0.75.

Cell culture and inhibitors

The human embryonic kidney cell line HEK-293T and HeLa cell line were maintained in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100mg/ml streptomycin at 37°C with 5% (vol/vol) CO_2. The class III sirtuin (SIRT) inhibitor nicotinamide and HDAC inhibitor Trichostatin A (TSA) were purchased from Sigma.

Plasmids and antibodies

The cDNA of GAPDH, ACTN1, NPM1 and DDX5 were amplified by PCR and subcloned into 3×FLAG vector (Sigma). The following antibodies were used in the experiments: Pan-Kcr (PTM-Biolabs 501), FLAG and HA (Sigma F1804 and H3663), H3 (Cell Signaling Technology 5246), CBP (Cell Signaling Technology 7389), HDAC1 (Cell Signaling Technology 5356), HDAC3 (Cell Signaling Technology 3949), NPM1 (Santa Cruz Sc-47725), FHL1 (Proteintech 10991-1-AP) , ACTN1 (Abcam ab68194), ITGB1(Abcam ab24693), Vinculin(Abcam ab129002), ERK2(Abcam ab32081) and CDK1(Cell Signaling Technology 9116).

Co-immunoprecipitation

Cells were lysed in NP40 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10 mM sodium butyrate) containing protease inhibitors for 30 min on ice. Then, lysates were incubated with the relevant antibody overnight at 4°C. And 50μl

protein A/G agarose beads were added and incubated for 2 h at 4°C. After washed with NP40 buffer for three times, the immunoprecipitated complexes were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the indicated antibodies.

Immunoflurescence

Cells were cultivated on coverslips prior to experiment. Cells were pre-washed with PBS for twice and fixed with 4% paraformaldehyde for 15 min at room temperature. Then the cells were permeabilized with 0.1% NP40 for 15 min. Permeabilized cells were blocked with 5% BSA in PBS for 1h. After washed with PBS three times, cells were incubated overnight at 4°C with pan-Kcr antibody. After washed with PBS for three times, cells were incubated with FITC-goat anti-rabbit IgG for 1 h at 4°C. Finally, cells were stained with DAPI and mounted to glass slides. Microscopic images were captured by confocal laser-scanning microscope (Carl Zeiss LSM780, Germany).

Immunohistochemical staining

Tissue paraffin sections were deparaffinized in xylene and rehydrated gradually. Then the sections were incubated in 3% H_2O_2 for 30 min to quench endogenous peroxidases. To retrieve antigen, the sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 20 min. After incubating with pan-Kcr antibody at 4°C overnight, followed by washing with PBS for three times, then the PV6001 2-step plus Poly-HRP anti-rabbit IgG detection system (Zhong Shan Jin Qiao, Beijing, China) was applied. Finally the detection was performed by streptavidin-biotin-peroxidase method.