ZZEF1 is a histone reader and transcriptional coregulator of Krüppel-like factors

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METHODS

Materials

Human ZZEF1 iso5 cDNA (NCBI Gene ID 23140), KLF3 cDNA (NCBI Gene ID 51274), KLF4 cDNA (NCBI Gene ID 9314), KLF6 cDNA (NCBI Gene ID 1316), KLF9 cDNA (NCBI Gene ID 687), and Sp1 cDNA (NCBI Gene ID 6667) were cloned into pENTR3C, and subsequently cloned into pCAG-Myc or p3Flag destination vectors using Gateway techniques (Invitrogen). The cDNA encoding the ZZ1 domain (amino acids 1781-1828) and ZZ2 domain (1830-1877) of human ZZEF1 were cloned into the pGEX-6P-1 vector (Novagen). Point mutations were generated using a site-directed mutagenesis kit (Stratagene). Histone peptides bearing different modifications were synthesized at the CPC and Scilight Biotechnology. Anti-ZZEF1 (ab174652) antibody was obtained from Abcam. Anti-GST (sc-459, 1:1000) antibody was from Santa Cruz. Anti-Flag (M2, 1:5000) was from Sigma. The shRNAs targeting human ZZEF1 and KLF9 were obtained from Sigma. shRNA sequences of ZZEF1 are #1, 5'- AGACTGGCCATCAACGATTAA; and #2, 5'- TTCACACTGGATTCGTTTAAA. shRNA sequences of KLF9 are 9#1, 5'- GCTTTGTATGAGTTGTACTTT; and #2, 5'- CCCAGTGTCTGGTTTCCATTT.

Protein expression and purification

The human ZZEF1 ZZ domains (amino acids 1781-1828 for ZZ1 and 1830-1877 for ZZ2) were cloned into a pGEX-6P-1 vector and expressed in BL21 Rosetta 2 cells. Protein production was induced with 0.2 mM IPTG and incubated overnight at 18 °C in Luria broth (LB) supplemented with 0.05 mM $ZnCl_2$. The GST-ZZ proteins were purified on glutathione Sepharose 4B beads (Amersham) in binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40, 1mM PMSF, plus protease inhibitors (Roche)) and eluted by 100 mM Tris pH 8.0 containing 10 mg/ml reduced glutathione (Sigma). All ZZ domain mutant proteins were expressed and purified as the

WT protein. For NMR experiments, wild-type and mutant ZZEF1 (aa 1826-1877) with an N-terminal SUMO fusion cloned into pDEST17 vector were expressed in BL21(DE3)RIL cells in ^{15}N-labled M9 minimal media or LB media. Cultures were grown to OD_{600} of 0.8 and induced with 0.5 mM IPTG for 18 hours at 16 °C. His-SUMO-tagged constructs were bound to HisPur Ni-NTA agarose beads (Thermo Scientific) in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 2 mM DTT, and then washed with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM DTT. His-SUMO-tag was cleaved off overnight at 4 °C with ULP1 protease. Unlabeled samples were further purified by FPLC on a HiPrep 16/60 Sephacryl S-100 size-exclusion column using 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM DTT.

Peptide pulldown assays

1 µg biotinylated histone peptides with different modifications were incubated with 1 µg GST-fused ZZEF1 ZZ domain in binding buffer (50mM Tris-HCl pH7.5, 300mM NaCl, 0.1% NP-40, and 1 mM PMSF) overnight with rotation at 4 °C. Streptavidin magnetic beads (Pierce) were added to the mixture, and the mixture was incubated for 1 h with rotation at 4 °C. The beads were then washed three times using a magnetic stand and the bound proteins were analyzed using SDS-PAGE and Western blotting.

NMR experiments

NMR experiments were carried out at 298K on a Varian INOVA 600 MHz spectrometer equipped with a cryogenic probe. NMR samples contained 0.1 mM uniformly ^{15}N-labeled WT or mutant ZZEF1 in 20 mM Tris-HCl (pH 7) buffer supplemented with 150 mM NaCl, 2 mM DTT, and 10% D_2O. Binding was characterized by monitoring chemical shift perturbations in the proteins induced by H3 peptides (aa 1-12) (synthesized by Synpeptide).

Fluorescence spectroscopy

Spectra were recorded at 25 °C on a Fluoromax-3 spectrofluorometer (HORIBA). The samples containing 1 μM H1877W ZZEF1 (aa 1826-1877) in a buffer composed of 20 mM Tris (pH 6.8), 150 mM NaCl, and 1 mM DTT and progressively increasing concentrations of the peptides were exited at 295 nm. Emission spectra were recorded between 330 and 360 nm with a 0.5 nm step size and a 1 s integration time. The K_d values were determined using a nonlinear least-squares analysis and the equation:

$$\Delta I = \frac{\Delta I_{max}\left(([L] + [P] + K_d) - \sqrt{([L] + [P] + K_d)^2 - 4[P][L]}\right)}{2[P]}$$

where [L] is the concentration of peptide, [P] is the concentration of the protein, ΔI is the fluorescence intensity, and ΔI_{max} represents the change in fluorescence. K_d is the average of at least three experiments.

Co-immunoprecipitation

HEK293T cells were transfected together with pCAG-Myc-ZZEF1 iso5 and p3Flag control vector, or with p3Flag-H3, or with p3Flag-KLF3, or with p3Flag-KLF4, or with p3Flag-KLF63, or with p3Flag-KLF9, or with p3Flag-Sp1. 48 h later, cells were lysed in cell lysis buffer (50mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1mM DTT, 1 mM PMSF, plus protease inhibitors) and sonicated. Anti-Flag M2-conjugated agarose beads (Sigma) were incubated with the lysates overnight at 4 °C. The beads were then washed 4 times with cell lysis buffer, and the bound proteins were eluted in SDS buffer and analyzed by western blotting.

Cell culture, viral transduction, and cell growth assay

Human HEK293T cells and human lung cancer cell line H1299 were maintained in DMEM (Cellgro) supplemented with 10% fetal bovine serum (Sigma), 2 mM $_{L}$-glutamine and 100 U ml^{-1} penicillin/streptomycin. Human lung cancer cell lines A549 were cultured in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (Sigma), 2 mM $_{L}$-glutamine and 100 U ml^{-1} penicillin/streptomycin. Lentiviral transduction was performed as described previously. Briefly, HEK293T cells were co-transfected with pMD2.G, pPAX2 (Addgene) and pLKO shRNA, and then viral supernatants were collected and filtered using 0.45 μm filters after 48 h. For infections, cells were incubated with viral supernatants in the presence of 8 μg ml^{-1} polybrene. After 48, the infected cells were selected with puromycin (2 μg ml^{-1}) for pLKO clones for 3-4 days before experiments.

After puromycin selected, the surviving cells were seeded in wells of 24-well plate (1,200 cells/well) at 37 °C in an atmosphere of 5% CO_2. The numbers of cells were counted daily for 7 days using a Celigo Imaging Cytometer (Nexcelom).

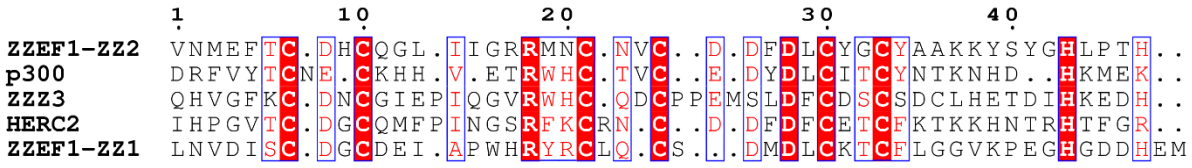
RNA extraction and real-time PCR

Total RNA was extracted using an RNeasy plus kit (Qiagen) and reverse-transcribed using an iScrip reverse transcription kit (Bio-Rad). Quantitative real-time PCR (qPCR) analyses were performed using PowerUp SYBR Green PCR Master Mix (Applied Biosystems) and Bio-Rad Real-Time System instrument. Gene expression were calculated following normalization to GAPDH levels using the comparative Ct (cycle threshold) method. Statistical differences were calculated using a two-way unpaired Student's *t*-test. The primer sequences for qPCR are listed in Supplementary Table 4.

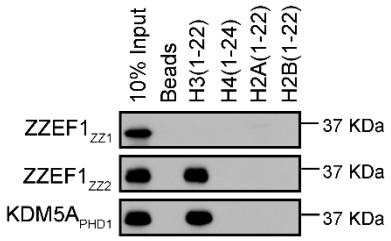
RNA-seq and ChIP-seq data processing

The paired end reads from raw data were mapped to human hg38 genome by HISAT2 (v2.1.0) with up to 1 alignment per read. Gene expression RPKMs and DEGs (q-value<0.05) were generated by cufflinks (v2.2.1). Motifs were scanned +/- 1 kbp around TSS by Homer findMotifsGenome (v4.9.1) with all TSS as background. DAVID (v6.8) was used to calculate enriched GO terms and KEGG pathways for up and down-regulated genes, the cut off was set to FDR<0.05.

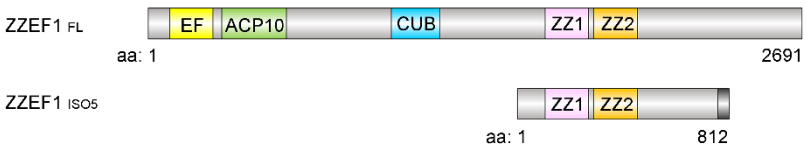
The bed files of KLF6 and KLF9 ChIP-seq in cell lines were downloaded from ENCODE (https://www.encodeproject.org). The genomic regions proportion analysis was done by Homer annotatePeaks (v4.9.1). GSEA (v4.0.3) was used to compare preference of KLF peaks on ZZEF1 regulated genes.



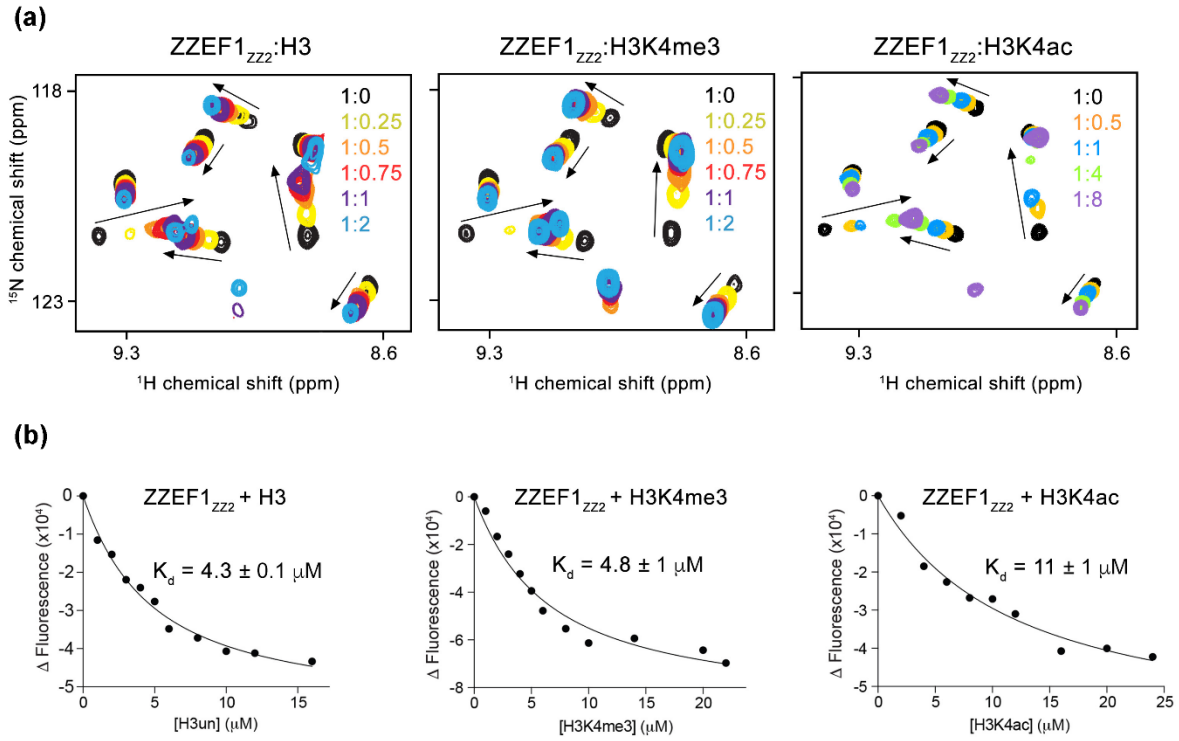
Supplementary Figure S1. Sequence alignment of ZZ domains from ZZEF1, p300, ZZZ3 and HERC2. Identical residues are shaded in red, conserved residues are indicated in red and blue box.



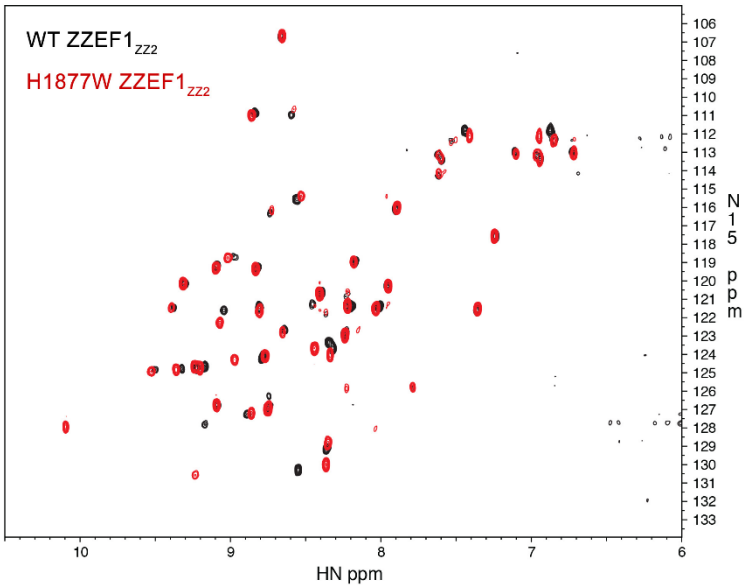
Supplementary Figure S2. The ZZEF1$_{ZZ2}$ domain binds to histone H3 tail. Western blot analysis of peptide pull-downs of GST-tagged ZZEF1$_{ZZ1}$ and ZZEF1$_{ZZ2}$ with the indicated histone peptides. Peptide pull-downs of KDM5A$_{PHD1}$ are shown for comparison.



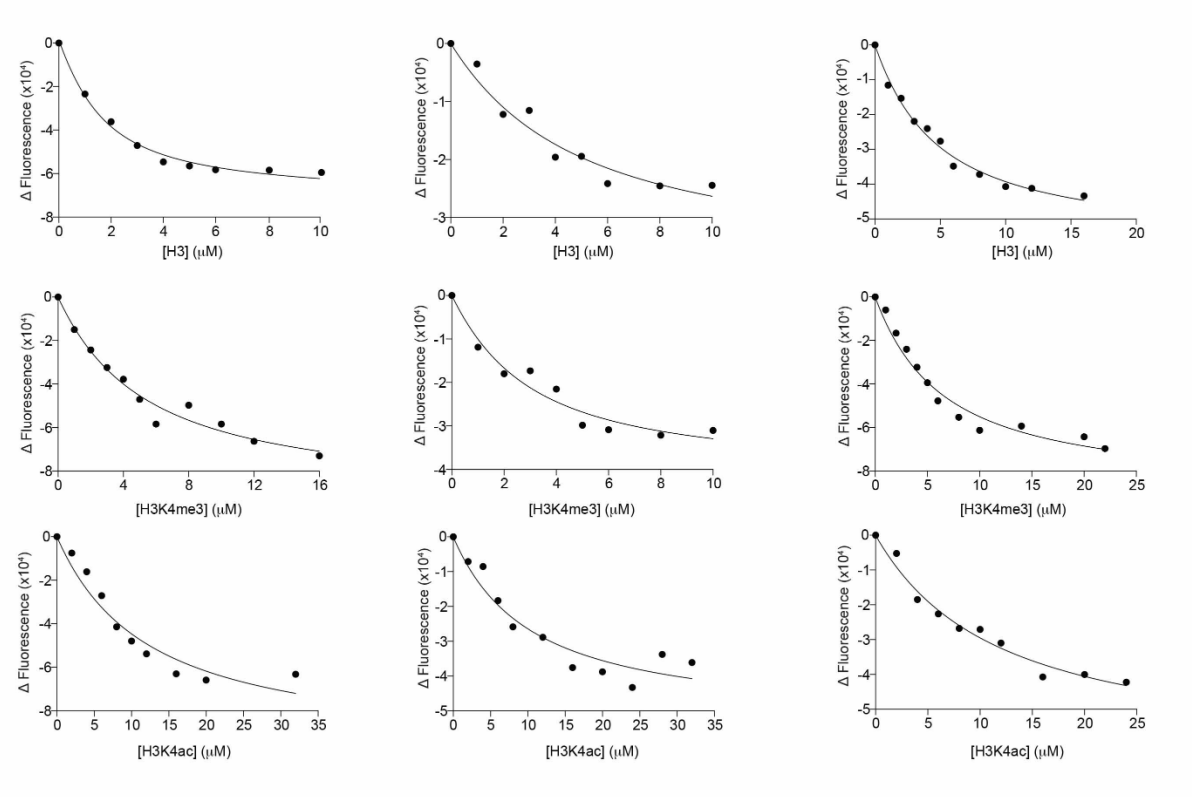
Supplementary Figure S3. Schematic representation of ZZEF1 full-length protein and isoform 5. Color schematics are the same as Fig. 1a.



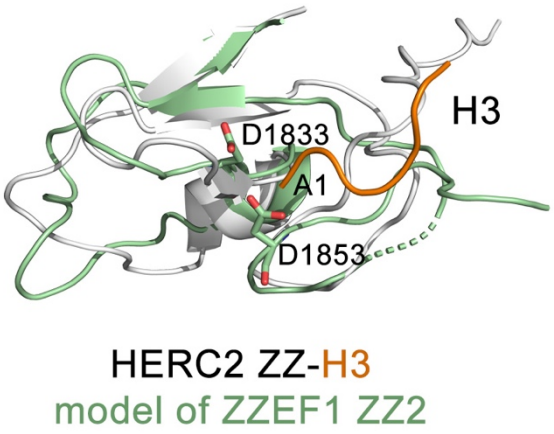
Supplementary Figure S4. Binding of ZZEF1 $_{ZZ2}$ to histone H3 was observed by NMR and tryptophan fluorescence experiments. **a,** Overlays of $^{1}H,^{15}N$ HSQC spectra of ZZEF1$_{ZZ2}$ with increasing amounts of unmodified H3(aa 1-12), H3K4me3 and H3K4ac peptides. Spectra are color coded according to the molar ratio of ZZEF1$_{ZZ2}$: H3 peptide. **b,** Representative binding curves used to measure binding affinity of ZZEF1$_{ZZ2}$ (H1877W) to the indicated H3 peptides by tryptophan fluorescence.



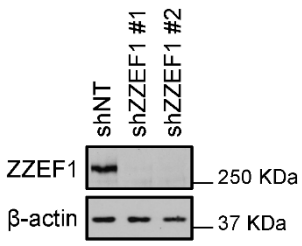
Supplementary Figure S5. The H1877W mutation does not affect the ZZEF1$_{ZZ2}$ fold. Overlay of the ^{1}H,^{15}N HSQC spectra of WT ZZEF1$_{ZZ2}$ (black) and ZZEF1$_{ZZ2}$ H1877W (red).



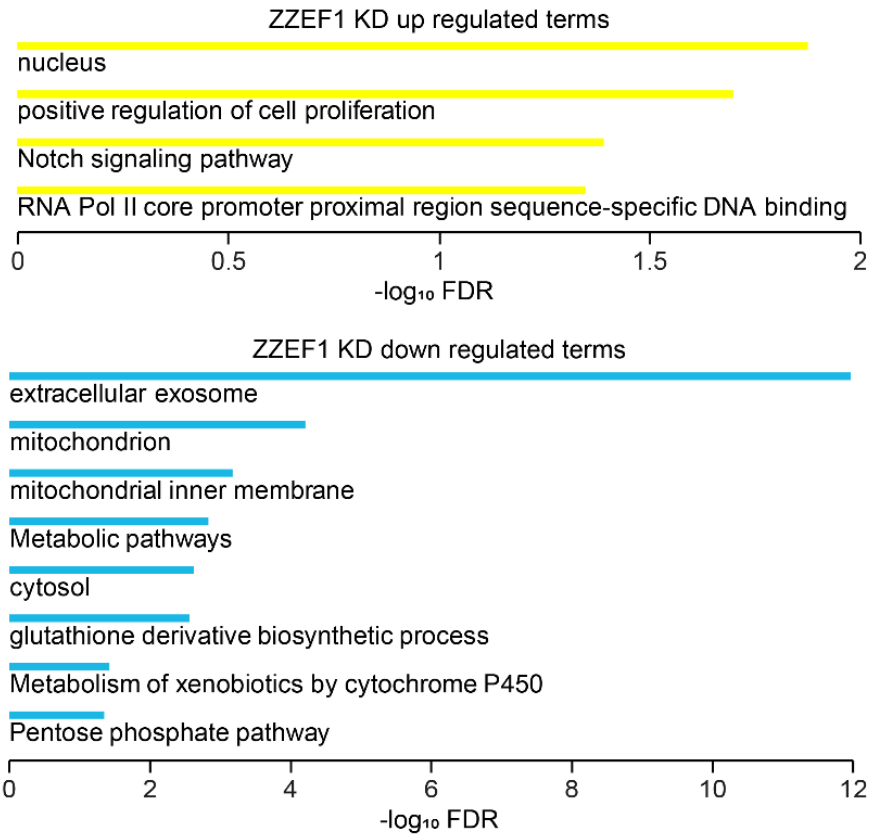
Supplementary Figure S6. ZZEF1$_{ZZ2}$ binds to histone H3 tail and tolerates PTMs on H3K4. Three independent replicates of tryptophan fluorescence measurements of binding of ZZEF1$_{ZZ2}$ (H1877W) to the unmodified H3(aa 1-12), H3K4me3 and H3K4ac peptides.



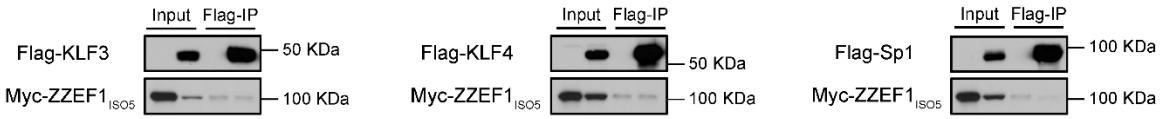
Supplementary Figure S7. A model of ZZEF1$_{ZZ2}$ binding to unmodified H3 tail. Overlay of the structure of the ZZ domain of HERC2 (grey) in complex with H3 (orange) (PDB ID: 6WW4) and the model of the ZZ domain of ZZEF1 (green). The two Aspartic acid residues (D1833 and D1853) important for H3 A1 binding are shown in stick.



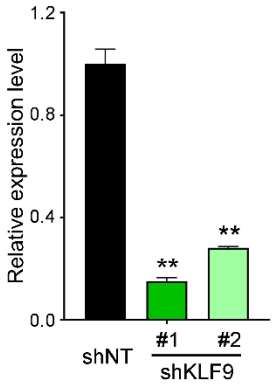
Supplementary Figure S8. Western blot analysis showing ZZEF1 shRNA KD efficiency in H1299 cells. β-actin is shown as a loading control. shNT: non-targeting control shRNA.



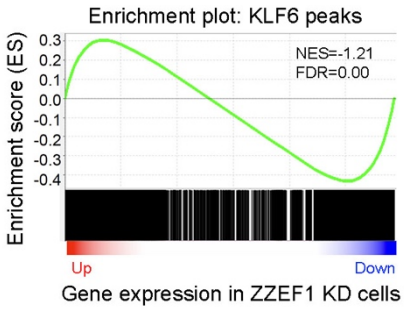
Supplementary Figure S9. Gene ontology (GO) analysis of differential expressed genes in ZZEF1 KD cells compared with control cells.



Supplementary Figure S10. ZZEF1 does not bind KLF3, KLF4 or SP1. Western bolt analysis of Flag co-IP using the M2 anti-Flag antibody in cells expressing Flag-KLF3, KLF4, Sp1 and Myc-tagged ZZEF1 iso5.



Supplementary Figure S11. qRT-PCR analysis of *KLF9* gene in control (shNT) and *KLF9* shRNA KD H1299 cells. Error bars indicate SD of three replicates. * $p < 0.05$; ** $p < 0.01$ (Student's *t*-test)



Supplementary Figure S12. GSEA analysis showing that down-regulated genes in ZZEF1 KD cells are enriched for KLF6 occupancy. NES: normalized enrichment score, FDR: false discovery rate.