Elevated histone H3 acetylation and loss of the Sp1–HDAC1 complex de-repress the GM2-synthase gene in renal cell carcinoma

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List of Supporting Information:

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Supporting Information Table S1: List of primers.

A) For quantitative real time PCR

Abbreviation	**Forward**	**Reverse**
GM2-synthase	5' –TTT GAC CCT GCA GAG CTG-3'	5' –CTG AAC TTC CAC ACC CTG TAG-3'
Sp1	5' –GTG AAA TCC CAT AGC CCT TAC C-3'	5' –CCA GAG GAT GAC AGA CTT AGG A-3'
HDAC1	5'-TAT CAA AGG ACA CGC CAA GT–3'	5' –TCA TTA GGG ATC TCC GTA TCC A–3'
GAPDH	5' –ACA ACT TTG GTA TCG TGG AAG G– 3'	5' –GCC ATC ACG CCA CAG TTT C– 3'
Beta Tubulin	5' –CTG TCC TGG ATG TGG TAC G–3'	5' –ATT CAT GAT GCG ATC AGG GTA TTC– 3'

B) For RT-PCR

Abbreviation	**Forward**	**Reverse**
GM2-synthase	5'-AAG CTA CCA GAC CAA CAC AGC AGA-3'	5'-GGC AGC TTC AGT TTG GAT GCA TGA-3'
Beta actin	5'-ACG TTG CTA TCC AGG CTG TGC TAT-3'	5'-ACT CCT GCT TGC TGA TCC ACA TCT-3'

C) For ChIP assay:

Abbreviation	Forward	Reverse
GM2-synthase +38 to +187 (Region P)	5' –CGC ATT CCC CGC GCG GAG CC– 3'	5' –AGC CCC GGG GCA AAG CCG GG– 3'
GM2-synthase +778 to +987 (Region Q)	5' –CAT TAA GAT GCC AGG CCT CG– 3'	5' –ACA CAT CGC GCT AGG GTT TG– 3'
GM2-synthase +2878/+3037 (Region R)	5' –CTA ACC CTG CAC TCT CCA ATC– 3'	5' –TAG TTG CCT GTT GAG TTG GTC– 3'

D) For Luciferase assay:

Abbreviation	Forward	Reverse
GM2-synthase +38 to +187 (Region P)	5' –CGC ATT CCC CGC GCG GAG CC– 3'	5' –AGC CCC GGG GCA AAG CCG GG– 3'
Sp1 site SDM	5' –CGG TGC GAA GAG CC*A AA*C GAC GGC CAG AGC– 3'	5' –GCT CTG GCC GTC GTT T*GG C*TC TTC GCA CCG– 3'

E) For EMSA:

Abbreviation	Forward	Reverse
Sp1 site at Region P (22 bp)	5' –AAG AGC CGG GCG GCG GCC AGA G– 3'	5' –CTC TGG CCG CCG CCC GGC TCT T– 3'
Consensus Sp1 binding oligo (22bp)	5' – ATT CGA TCG GGG CGG GGC GAG C– 3'	3' – TAA GCT AGC CCC GCC CCG CTC G– 5'

F) For DAPA:

Abbreviation	Forward	Reverse
Sp1 wild type probe	5' –biotin-TGC GGT GCG AAG AGC CGG GCG GCG GCC AGA GCC CTC CCC G– 3'	5' –CGG GGA GGG CTC TGG CCG CCG CCC GGC TCT TCG CAC CGC A– 3'
Sp1 SDM probe	5' –biotin-TGC GGT GCG AAG AGC CAA ACG GCG GCC AGA GCC CTC CCC G– 3'	5' –CGG GGA GGG CTC TGG CCG CCG TTT GGC TCT TCG CAC CGC A– 3'

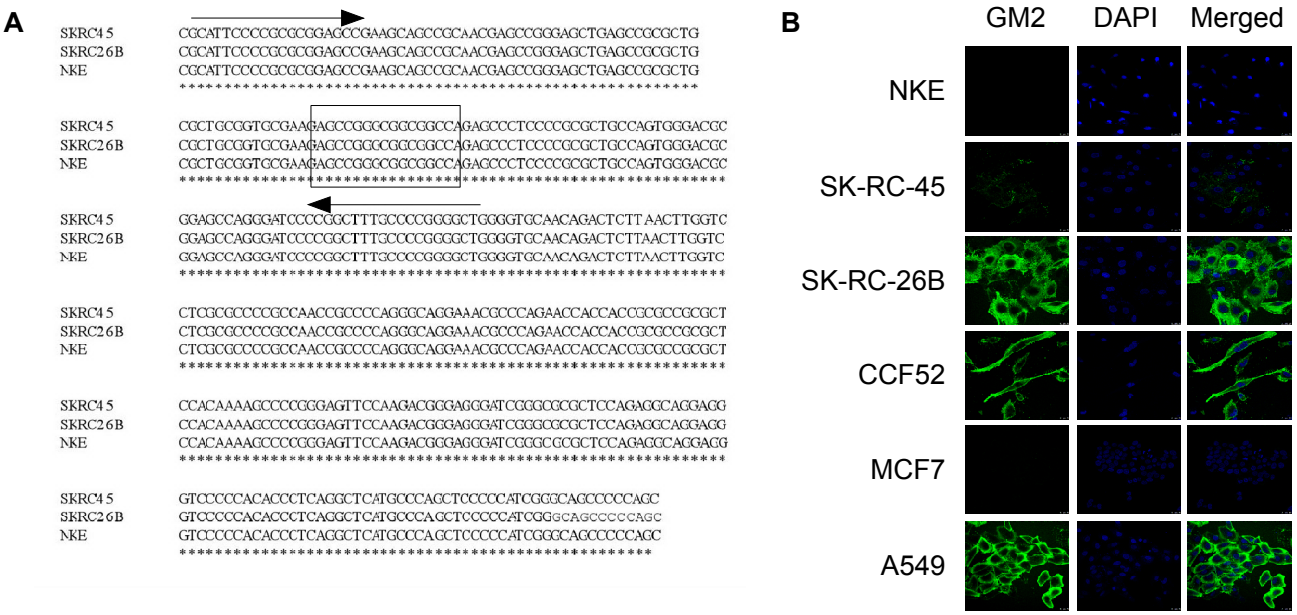
G) For Sp1 plasmid SDM:

Abbreviation	Forward	Reverse
pSp1 K703 SDM	5' –GCT TCA TGA GGA GTG ACC ACC TGT CAA *C*AC ATA TCA AGA CCC ACC AGA ATA AG– 3'	5' –CTT ATT CTG GTG GGT CTT GAT ATG T*G*T TGA CAG GTG GTC ACT CCT CAT GAA GC– 3'

H) For COBRA assay:

Abbreviation	Forward	Reverse
GM2-synthase for untreated genome DNA (-114 to +36)	5' –AGC AGG CAA TGG AAT GGA TG– 3'	5' –AGC GCC CCG GCG CCT TCT AG– 3'
GM2-synthase for untreated genome DNA (+38 to +187)	5' –CGC ATT CCC CGC GCG GAG CC– 3'	5' –AGC CCC GGG GCA AAG CCG GG– 3'
GM2-synthase for bisulphite treated genome DNA (-73 to +158)	5' –GAT GTT AGT GTT TGG AGG TTA AGG A– 3'	5' –CTA ACT CCA CAT CCC ACT AAC AAC A– 3'
T7 forward	5’ –TAA TAC GAC TCA CTA TAG GG -3’	

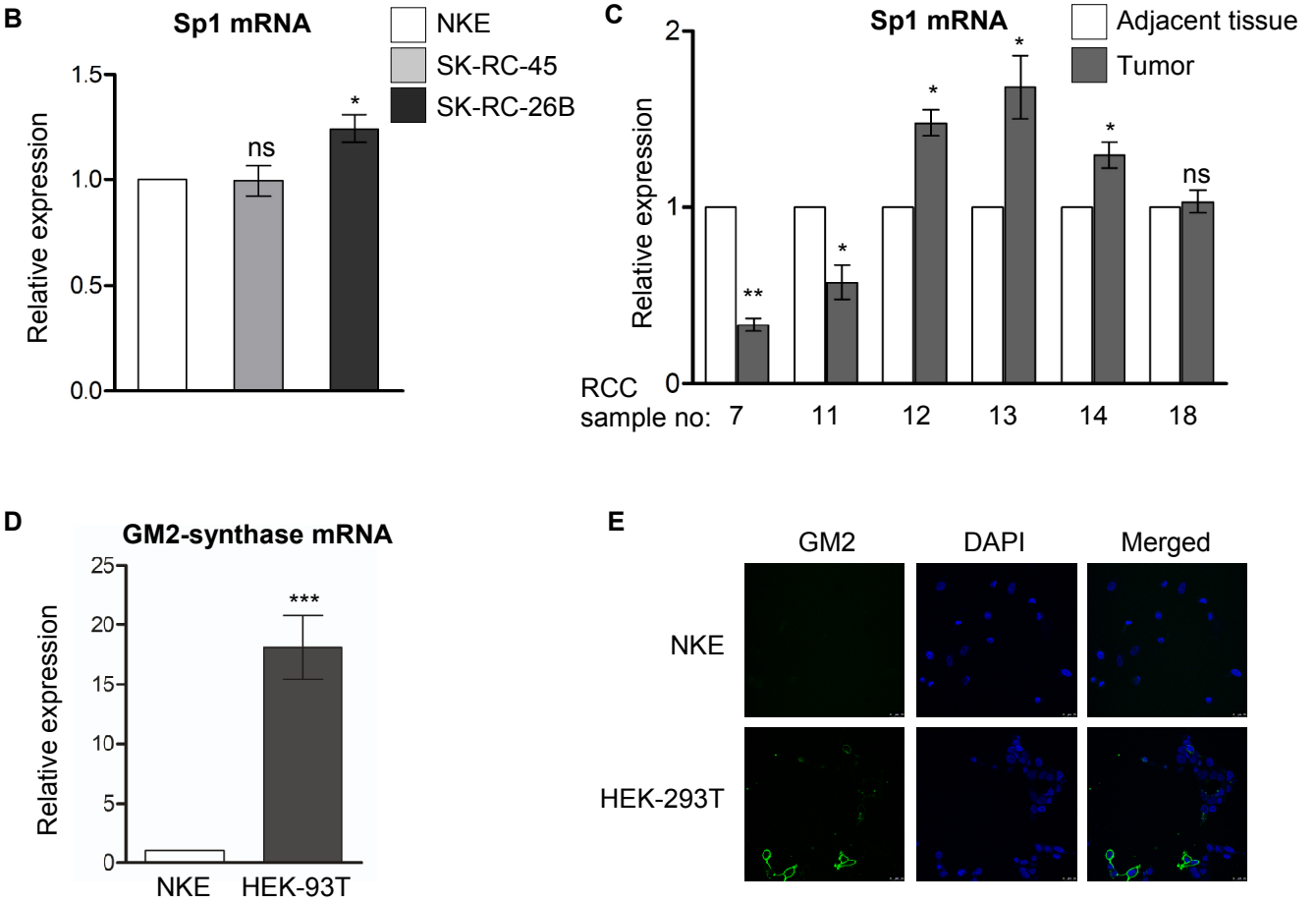
Supporting Information S1



SUPPORTING INFORMATION S1. *A,* DNA sequence showing identical +38 to +387 region downstream of the transcription start site (TSS) of GM2-synthase gene in NKE, SK-RC-45 and SK-RC-26B cell lines. The arrows represent the region +38 to +187 of GM2-synthase gene encompassing the Sp1 binding site (highlighted with the rectangular box) *B,* Ganglioside GM2 expression profile in indicated cell lines. Immuno-staining of GM2 in indicated cell lines was performed with hamster anti-human GM2 antibody. Nucleus was stained with DAPI.

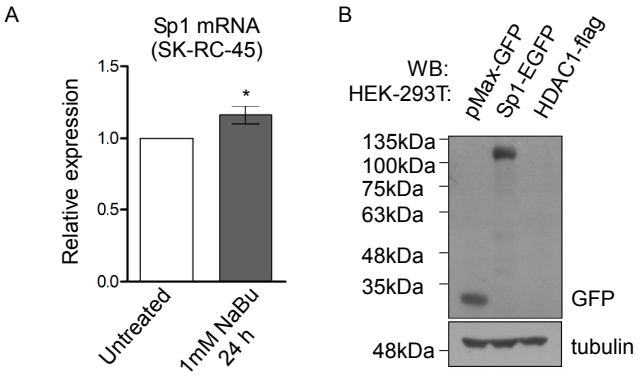
A

Matrix	Detailed Matrix Information	Tissue	Strand	Matrix similarity	Sequence
V$SP1.03	Stimulating protein 1, ubiquitous zinc finger transcription	ubiquitous	+	0.916	gagccGGGCggcggcca



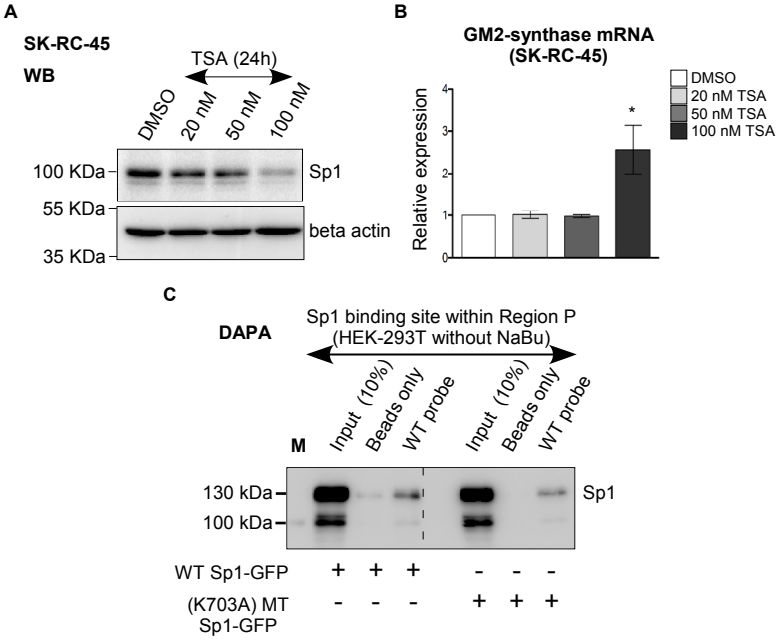
SUPPORTING INFORMATION S2. *A,* Genomatix MatInspector based analysis of the +109/+125 Sp1 binding site on GM2-synthase promoter. *B, C,* Sp1 mRNA expression profile in indicated cell lines and tissue samples. Total RNA was isolated from indicated cell lines and tissues and reverse-transcribed. cDNAs were subjected to quantitative real time PCR (qPCR) using Sp1 primer. Relative expression values were normalized to the beta tubulin transcripts levels. The data represent three independent determinations (average ± SEM, Students t test, ns: not significant, **p<0.01). *D,* HEK-293T cells express high GM2-synthase mRNA over NKE. qPCR was performed with GM2-synthase primer. Relative expression values were normalized to the GAPDH transcripts levels. The data represent three independent determinations (average ± SEM, Students t test, ***p<0.001). *E.* Ganglioside GM2 expression profile in indicated cell lines. Immuno-staining of GM2 in indicated cell lines was performed with hamster anti-human GM2 antibody. Nucleus was stained with DAPI.

Supporting Information S3

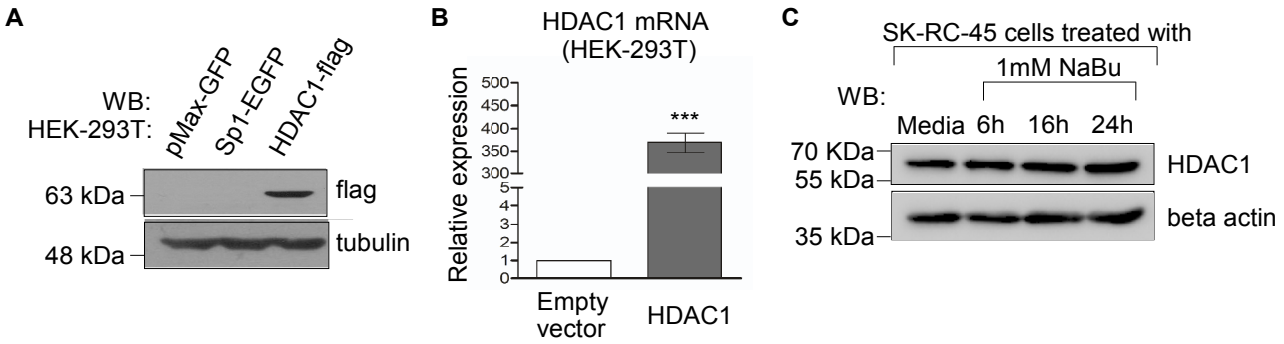


SUPPORTING INFORMATION S3. *A,* Sp1 mRNA expression shows no decrease upon NaBu treatment. Total RNA was isolated from SK-RC-45 cells treated with 1mM NaBu up to 24 h and reverse-transcribed. cDNAs were subjected to quantitative real time PCR (qPCR) using Sp1 primer. Relative expression values were normalized to the GAPDH transcripts levels. The data represent three independent determinations (average ± SEM, Students t test, ***$p<0.001$). *B,* Successful over-expression of Sp1 protein in HEK-293T cells. HEK-293T cells were transfected separately with pMax-GFP, pSp1-GFP and pHDAC1-Flag vectors for 60 h, and cell extracts were prepared followed by Western blot analysis with antibody against GFP to detect over expressed Sp1 of molecular weight 125 KDa. Tubulin was used as an endogenous control.

Supporting Information S4



SUPPORTING INFORMATION S4. *A.* TSA treatment on SK-RC-45 cells down regulates endogenous Sp1 protein expression. SK-RC-45 cells were treated with TSA at mentioned concentrations for 24 h, and cell extracts were prepared followed by Western blot analysis with antibodies against Sp1 and beta actin. *B,* TSA treatment in SK-RC-45 cells increases GM2-synthase transcription. SK-RC-45 cells were treated with TSA at mentioned concentrations for 24 h. Total RNA was isolated and reverse-transcribed. cDNAs were subjected to qPCR using GM2-synthase primer. Relative expression values were normalized to the house keeping gene GAPDH transcripts levels and represented as fold change with respect to DMSO treated cells as vehicle control. The data represent three independent determinations (average ± SEM, Students t test, *p<0.1). *C,* Relative binding of wild type (WT) Sp1 protein versus versus mutant (MT) Sp1 to the Sp1 binding site within Region P of GM2-synthase gene *in vitro*. DNA affinity precipitation assay (DAPA) with cell extract of HEK-293T cells which were transfected with either wild type (WT) Sp1 or K703A mutated (MT) Sp1 plasmids were incubated with 22 bp biotinylated probe encompassing the Sp1 binding site within Region P of GM2-synthase gene (WT probe). Proteins bound to the biotinylated probes were pulled down by streptavidin beads and probed for Sp1 protein using anti-Sp1 antibody by immunoblotting. The molecular weight of over expressed Sp1 which is tagged with GFP is 125 KDa. 'Beads only' panel was included as a negative control. M denotes protein marker.



SUPPORTING INFORMATION S5. *A,* Expression of Flag tagged HDAC1 vector in HEK-293T cells. HEK-293T cells were transfected separately with pMax-GFP, pSp1-GFP and pHDAC1-Flag vectors for 60 h, and cell extracts were prepared followed by Western blot analysis with antibodies against flag and tubulin. *B,* Increased HDAC1 mRNA levels in pHDAC1-flag transfected cells. Total RNA was isolated from empty vector or pHDAC1-flag vector transfected HEK-293T cells and reverse transcribed. qPCR was performed with HDAC1 primer. Relative expression values were normalized to the GAPDH transcript levels. The data represents three independent determinations (average ± SEM, Students t test, ***$p<0.001$). *C,* HDAC1 protein expression do not decrease in SK-RC-45 cells treated with NaBu. Cell extracts were prepared from SK-RC-45 cells treated with NaBu followed by Western blot analysis with antibodies against HDAC1 and beta actin.