STK33 Promotes Hepatocellular Carcinoma through binding to c-Myc

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Supplemental methods, supplemental figures and figure legends, supplemental tables.

Materials and Methods

Cell cultures, plasmids and antibodies

HCC cell lines HepG2, Hep3B, Bel7402, SMMC7721, HKCL2, HKCL9 SK-HEP1, and immortalized human liver cell L02, were purchased from American Type Culture Collection (ATCC), and were cultured according to the instructions from ATCC. L02 and HepG2 cells were transfected with the plasmids of full-length, wild-type of STK33 or the plain vector, while HKCL2 and SMMC7721 were transfected with the plasmids of STK33 shRNA or control shRNA, Lipofectamine 2000 (Invitrogen). They were selected by 0.8 mg/ml G418 (GIBCO BRL) for two weeks before cells with stable overexpression or knockdown of STK33 pool were gotten for the subsequent assays. Fipofectamine 2000 was used for transfection in c-Myc knockdown and cells were collected for relevant experiments after 72 hours. STK33 plasmid was transfected in to HepG2/c-Myc shRNA cells for STK33 rescue, the transfected cells were then selected by 0.8 mg/ml G418 (GIBCO BRL) plus 0.6 mg/ml Zeocin (Sigma) for two weeks until stable STK33 overexpression with c-Myc knockdown cells pool were obtained.

Target sequences of human STK33 shRNA1: 5'-TTAGCAGCAAGTTAATGGC-3', STK33 shRNA2: 5'-GCATTAAAGCAGCGTATC-3'; target sequence of human c-Myc shRNA: 5'-TTGCTCCTCTGCTTGGACG-3', and shRNA non-sense control target sequence: 5'-TGAGCAGGCGCATGTGCTG-3', all the shRNA plasmids were obtained from Thermo. The plasmid of STK33 (MHS1010-98053352) and c-Myc (MHS1010-57504) were purchased from Thermo. Primary antibodies to STK33 (ab110090), and c-Myc (ab320720) were purchased from Abcam. Primary antibodies to α–tubulin were

purchased from Sigma. Second antibodies (anti-mouse IgG-HRP and anti-rabbit-HRP) were purchased from Sigma. MTT assay reagents were purchased from DingGuo Biotech.

Animals, Treatments, and Tissue Collection

STK33 floxed transgenic mice ($STK33Tg^{flox/flox}$) and knockout mice ($STK33KO^{flox/flox}$) were generated according to standard procedures. A CMV promoter vector with two loxP sites containing the sequence encoding the TAG stop site, was designed to code STK33 cDNA in $STK33Tg^{flox/flox}$ mice. For $STK33KO^{flox/flox}$ mice, phage clones containing mouse STK33 were isolated by screening of a 129/SvJ genomic library (Stratagene) with a probe corresponding to the 5' end of mouse STK333 cDNA. A targeting vector was designed to flank exon 1, containing the sequence with two loxP sites encoding for the ATG site. The floxed neomycin-resistance gene fragment was inserted into intron 1 of STK33. A 1.0–kilobase (kb) ClaI–BamHI fragment was used as the 5' homology region; a 2.5-kb XbaI–SalI fragment, which contains exon 1 of STK33, was inserted between the two loxP sites; and a 6.0-kb NotI–SacII fragment was used as the 3' homology region. The herpes simplex virus thymidine kinase gene was used for negative selection of clones with random integration. A total of 30 mg of SacII-linearized vector was electroporated into E14.1 embryonic stem cells. After positive and negative selection with G418 and ganciclovir, drug-resistant clones were picked up and were screened by PCR and southern blot analysis. These clones were individually microinjected into blastocysts derived from C57BL/6 mice and were transferred to pseudopregnant females. Matings of chimeric male mice to C57BL/6 female mice resulted in transmission of the floxed allele to the germline. $STK33Tg^{flox/flox}$ or $STK33KO^{flox/flox}$ mice were bred with transgenic mouse line carrying the Cre-ER^{T2} transgene under control of albumin promoter (Alb-Cre-

ER^{T2}) to generate the $STK33Tg^{flox/+,\ Alb\text{-}ERT2\text{-}Cre}$ or $STK33KO^{flox/+,\ Alb\text{-}ERT2\text{-}Cre}$, which were then intercrossed to generate $STK33Tg^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ or $STK33KO^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice.

Alb-Cre-ER^{T2} mice was generated as previously described [17]. All animals were housed in laboratory animal facilities at the Second Military Medical University Medical Center under a standard 12-h light/dark cycle with access to chow and water as libitum. Institutional Animal Care and Use Committee of the Second Military Medical University was approved for all experiments. All mice were euthanized after their experimental periods.

Eight-week-old male, $STK33Tg^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice were treated with TAM (6mg/mouse, IP), or with vehicle alone (corn oil, IP, referred as control) subcutaneously. To detect hepatocyte proliferation and liver weight/body ratio and tumor burst ratio, mice were killed with CO_2 and livers were collected 7 days and 10 months after the injection, respectively. For BrdU detection, 1mg/Kg BrdU were peritoneal injected into mouse 2 hours before sacrifice.

Luciferase reporter assay

To evaluate c-Mc-dependent transcriptional activity, luciferase reporter assay was performed with a pair of luciferase reporter constructs hTRET-Luc and M4mintK- (Promega). Cells were transiently transfected in triplicate with one of these luciferase reporters and pCMV-β-galactosidase (Promega) using Lipofectamine 2000 (Invitrogen). 48h after transfection, luciferase activity was determined using the Luciferase Assay

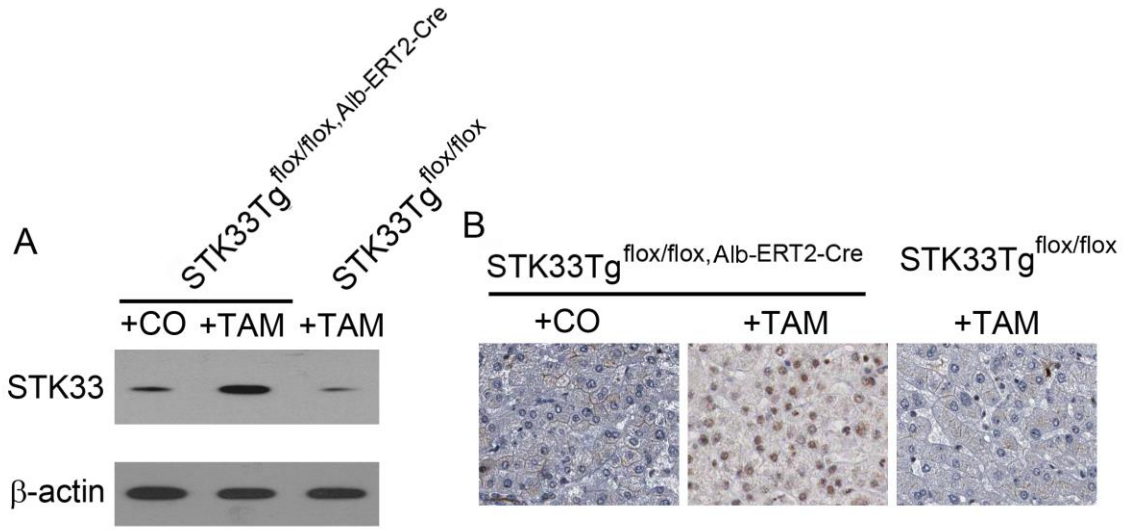
System Kit (Promega). β-galactosidase activity was determined using the Luminescent β-gal Detection Kit II (BD Clontech) as an internal control.

DNA preparation and detection of Ras point mutations

High molecular weight DNA was isolated from frozen HCC tumor samples or HCC cell lines. Selective amplification of the Ras gene sequence was done using a PCR technique. The nucleotide sequences of the primers used are listed in supplemental Table 3. The PCR was performed at 96 ^{o}C to denature the DNA (1 min), at 55 ^{o}C (NRAS), 57 ^{o}C (KRAS), 62 ^{o}C (HRAS) to anneal the primer (30 s), and at 72 ^{o}C to synthesize DNA (10 s to 1 min) using Taq DNA polymerase for 35–40 cycles in a DNA thermal cycler (Perkin-Elmer-Cetus). Amplified DNA samples were spotted onto nylon membranes (Hybond N?) for the hybridization analysis. All of the DNA isolated from the 251 tumor samples and the corresponding non-malignant liver tissues were screened for activated point mutations in codons 12, 13, and 61 of all three Ras genes using an oligonucleotide specific for the different sequences. The filters were prehybridized for 1 h at 55 ^{o}C in solution A (3.0 M tetramethylammonium chloride, 50 mM Tris-HCI, 2 HIMEDTA, 0.1% SDS, 59 Denhardt's solution, 100 fg/ml denatured herring sperm DNA), and hybridized for l h at 55 ^{o}C in the same solution with 5 pmol 32P-labeled probe. These filters were washed twice in 0.3 M NaCl, 0.02 M NaH2PO4, 2 mM EDTA and 0.1% SDS at room temperature for 5 min, and in solution A without Denhardt's solution and herring sperm DNA, once for 5 min at room temperature and twice for 10 min at 60 ^{o}C. These filters were then exposed to Kodak XAR5 film. Human cancer cell lines carrying Ras genes mutations were used as positive controls. The colon cancer cell lines: SW620 (KRAS

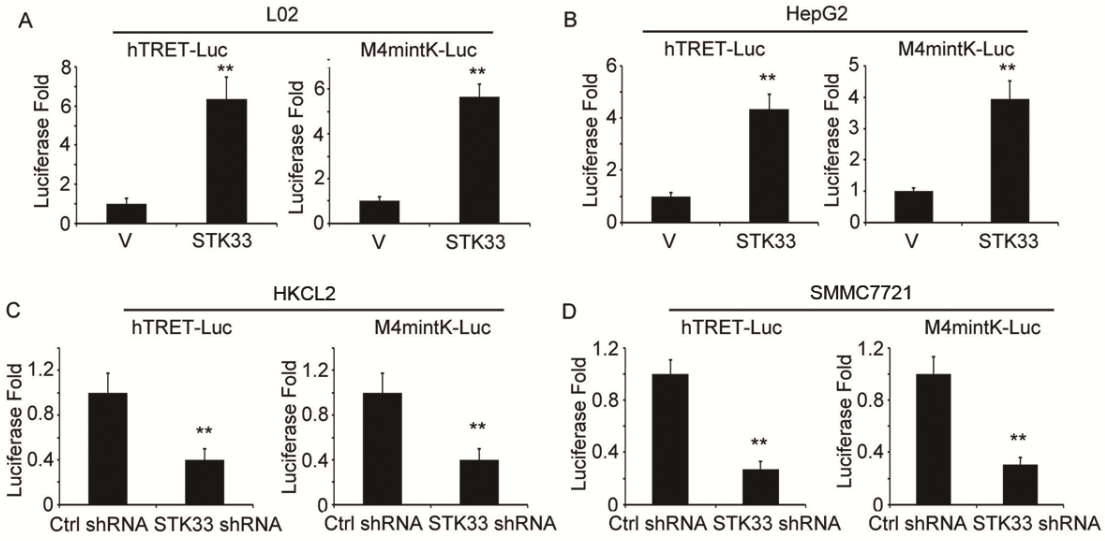
codon 12 GTT:Val), LSI80 (KRAS codon 12 GAT:Asp), and LOVO (KRAS codon 13 GAC:Asp) were obtained from ATCC.

S1



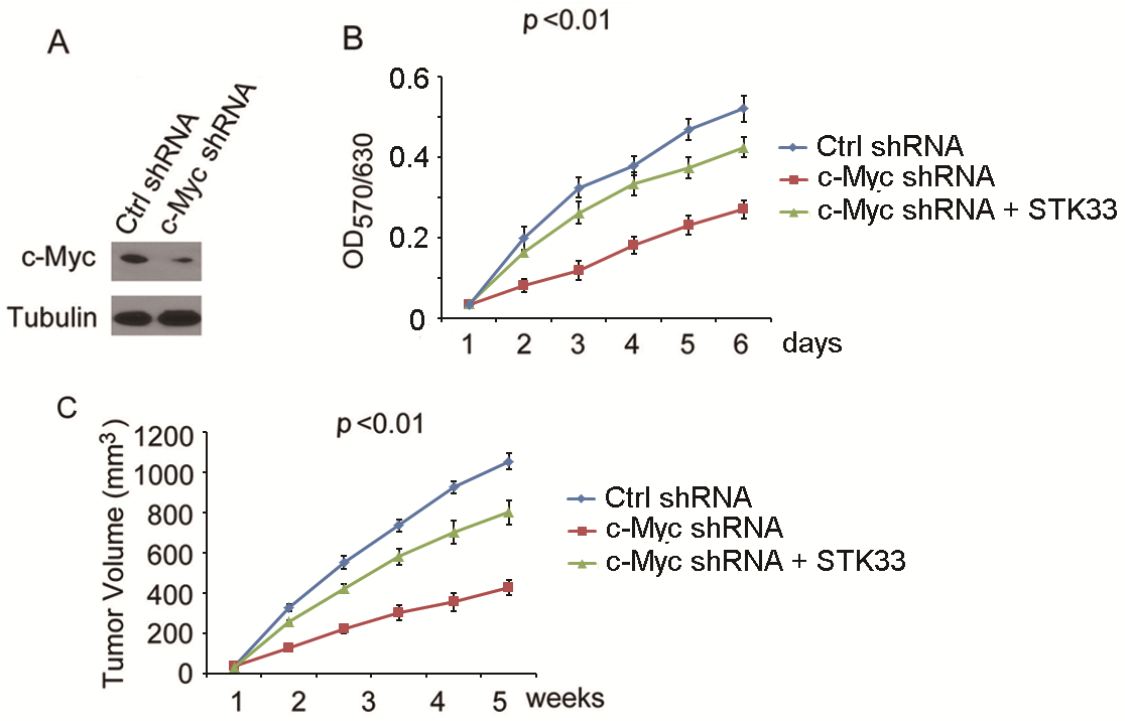
Supplemental Figure S1 Expression of STK33 in TAM-inducible liver specific STK33 transgenic mice. (A) Western blot analysis of STK33 using nuclear proteins isolated from STK33Tg$^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice treated with either TAM or corn oil and wild-typet C57 mice at day7 after TAM treatment. See Methods section for details. (B) Immunohistochemistry analysis of STK33. Sections were from STK33Tg$^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice treated with either TAM or corn oil and STK33 floxed littermate control mice treated TAM.

S2



Supplemental Figure S2 STK33 promotes c-Myc transcription activity. (A, B) Ectopic of STK33 enhanced c-Myc response hTRET-Luc and M4mintK-Luc reporter luciferase in L02 and HepG2 cells. (C, D) Knockdown of STK33 decreased c-Myc response promoter activities in HKCL2 and SMMC7721 cells. ** p <0.01. Results represent at least three separate experiments.

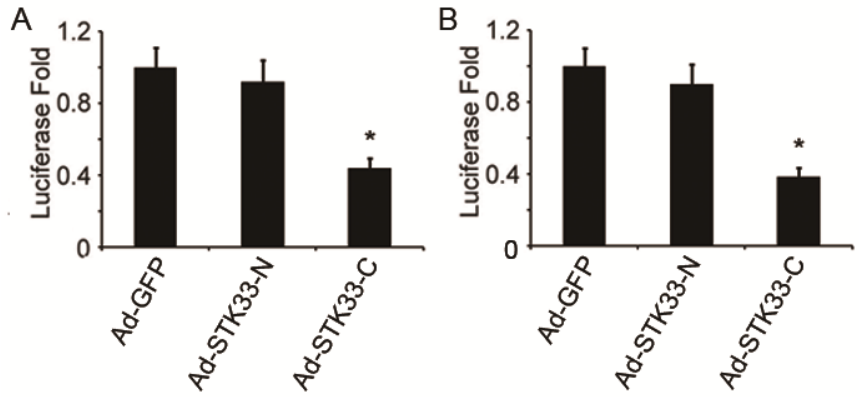
S3



Supplemental Figure S3 Over-expression rescues c-Myc-depletion in HCC cells. (A) c-Myc was knocked down in HepG2 cells. HepG2 cells were transfected with control shRNA (Ctrl shRNA) or c-Myc shRNA. c-Myc protein levels were detected by immunostaining with c-Myc antibody. Tubulin was as the loading control. (B) *In vitro* growth of HepG2 cells treated with nonsense control shRNA (Ctrl shRNA), c-Myc shRNA, or c-Myc shRNA plus STK33 overexpression, as measured by the MTT assay. (C) Average tumor volume in athymic nude mice subcutaneously inoculated with HepG2 cells treated with nonsense control shRNA (Ctrl shRNA), c-Myc shRNA, or c-Myc shRNA

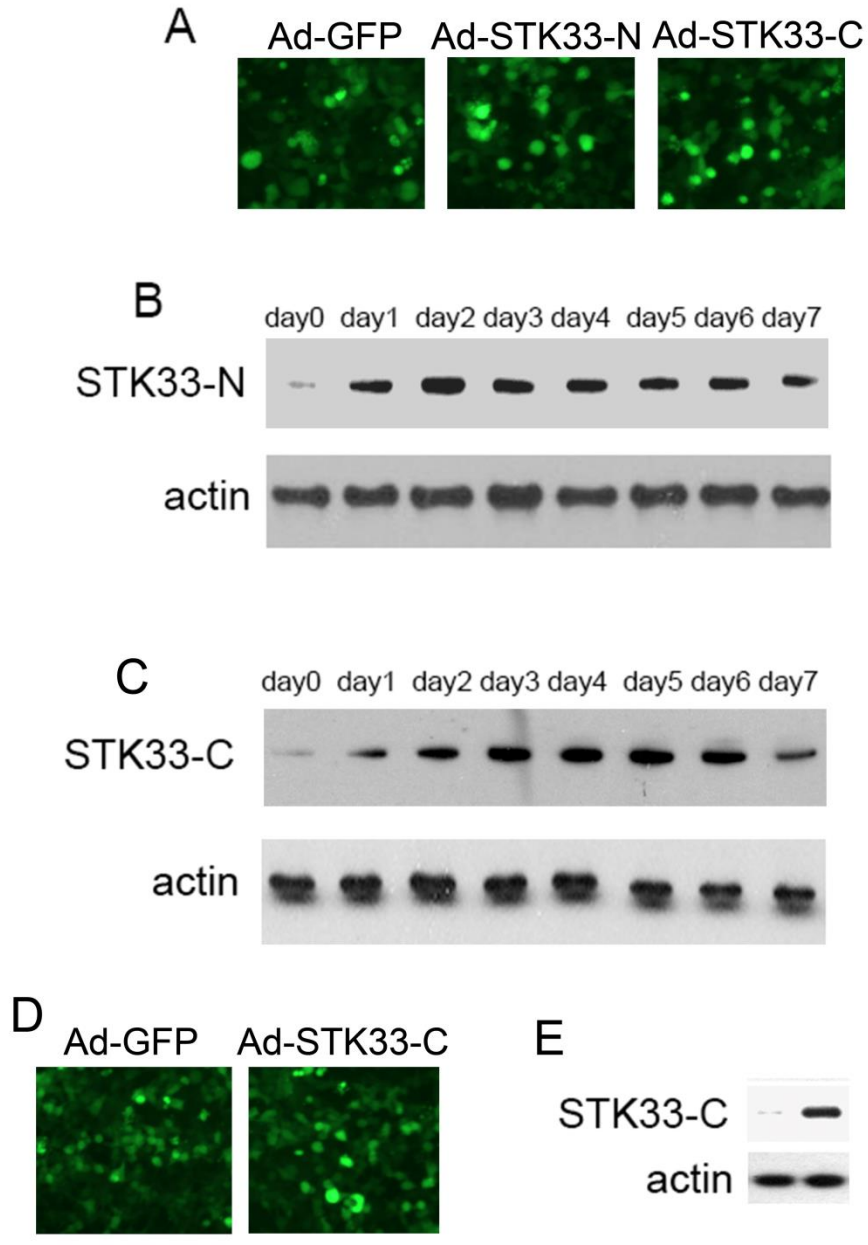
plus STK33 overexpression. Results represent at least three separate experiments for A and B. n=10mice/group for (C). Error bar represent ± S.D.

S4



Supplemental Figure S4 C-terminus of STK33 inhibits c-Myc transcription activity. Ectopic of STK33-C decreased c-Myc response hTRET-Luc (A) and M4mintK-Luc (B) reporter luciferase in SMMC7721 cells. * p <0.05. Results represent at least three separate experiments.

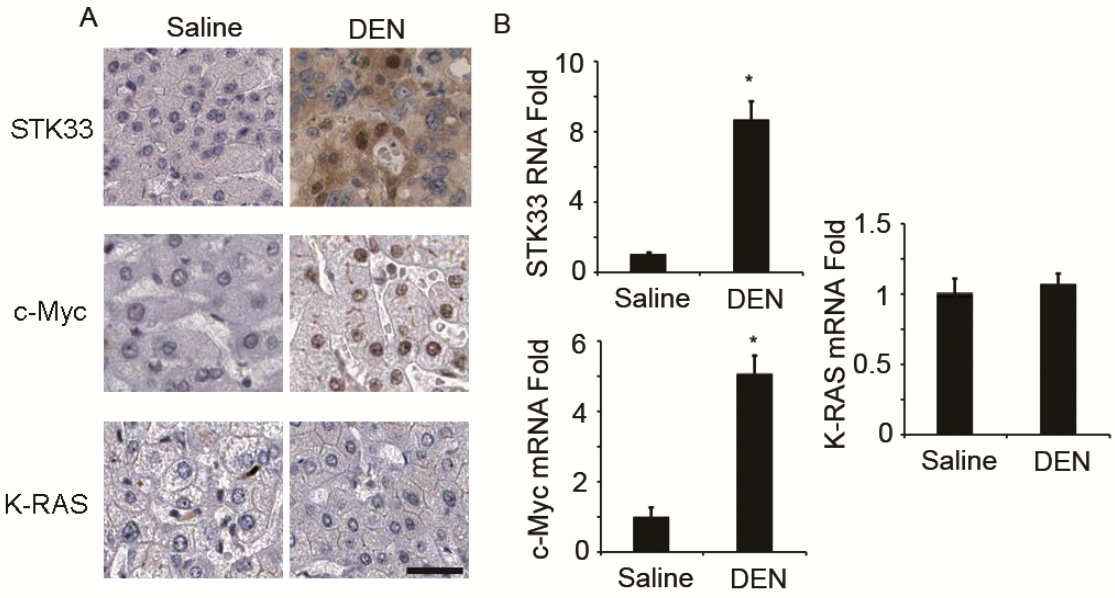
S5



Supplemental Figure S5 Expression of STK33-N and STK33-C in STK33Tg$^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice liver infected with relevant adenovirus. (A) Picture showed

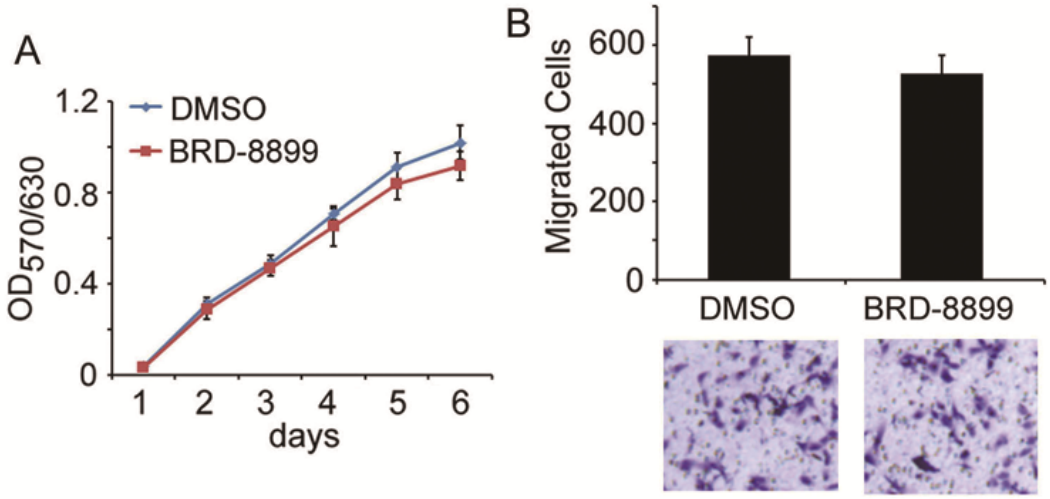
adenovirus infection rate (day3 after infection) in mice liver. (B, C) Adenovirus coding N-terminal of STK33 (STK33-N) or C-Terminal of STK33 (STK33-C) were injected into TAM inducible STK33Tg$^{flox/flox,\ Alb\text{-}ERT2\text{-}Cre}$ mice every 7 day. Liver cells were isolated, anti-Flag tag antibody was used to detect the expression of STK33-N and STK33-C after adenovirus injection at different time. (D) Picture showed adenovirus infection rate (day3 after infection) in DEN-induced mice tumor tissue. (E) Adenovirus coding C-Terminal of STK33 (STK33-C) or Ad-GFP vector control were injected into DEN-induced mice every 7 day, anti-Flag tag antibody was used to detect the expression of STK33-C after adenovirus injection (day3). Results represent at least three separate experiments.

S6



Supplemental Figure S5 Expression of STK33 in C57B/L mice liver after DEN treatment. (A) The level of STK33 in DEN-induced tumor tissues compared with liver tissue from mice treated with PBS (Saline), detected through immunostaining with the STK33 antibody. Representative pictures were shown. (B) The expression of STK33 was verified through qRT-PCR in DEN-induced tumor tissues compared with liver tissue from mice treated with PBS (Saline). For A-B, mice treated with 50 μg/g of DEN at age 20 days and killed 10 months later, the tumors were gotten for protein or mRNA analysis. Bar=50 μm.

S7



Supplemental Figure S7 STK33 kinase inhibitor BRD-8899 failed to inhibit STK33 induced cell proliferation and migration. (A) BRD-8899 did not inhibit proliferation of HepG2 cells with overexpressed STK33 (HepG2/STK33). (B) BRD-8899 did not inhibit migration of HepG2/STK33 cells.

Supplemental Table S1 Clinical Feature of HCC Patients

Feature	Number	Feature	Number
All case	251		
Age		Macrovascular invasion	
< 50	75	N	163
≥ 50	176	Y	88
Gender		Microvascular invasion	
Male	137	N	172
Female	114	Y	79
Clinical Stage		hepatitis surface B	
Stage I	35	N	216
Stage II	72	Y	35
Stage III	89	hepatitis surface C	
Stage IV	55	N	70
Tumor Size		Y	181
< 5cm	148		

≥ 5cm	103

Supplemental Table 2 Reported Ras gene mutations in HCC patients

	No. of patients	Ras gene mutation		
		KRAS	NRAS	HRAS
$STK33^{+}$	142	1 (codon 61), 2 (codon 13)	3 (codon 61)	0
$STK33^{-}$	109	2 (codon 61), 1 (codon 13)	2 (codon 61)	0

Supplemental Table 3 Ras gene primers used in this study

Gene/codon	Length (bp)	Sequence	
KRAS/12,13	108	Forward	GACTGAATATAAACTTGTGG
		Reverse	CTATTGTTGGATCATATTCG
KRAS/61	128	Forward	TTCCTACAGGAAGCAAGTAG
		Reverse	CACAAAGAAAGCCCTCCCCA
HRAS/12, 13	63	Forward	GACGGAATATAAGCTGGTGG
		Reverse	TGGATGGTCAGCGCACTCTT
HRAS/61	73	Forward	AGACGTGCCTGTTGGACATC
		Reverse	CGCATGTACTGGTCCCGCAT
NRAS/12, 13	109	Forward	GACTGAGTACAAACTGGTGG
		Reverse	CTCTATGGTGGGATCATATT
NRAS/61	103	Forward	GGTGAAACCTGTTTGTTGGA
		Reverse	ATACACAGAGGAAGCCTTCG