SUPPLEMENTARY MATERIALS

Supplementary Methods

Live-cell imaging

Negative control or AHNAK2 shRNA-tranduced CAKI-1 cells were cultured in glass bottom dishes (In Vitro Scientific, D35-20-1-N) for 12–18 h before imaging live at 37℃ in a confocal microscope (Nikon). Each image shown in video corresponds to a 8-min interval. Data were analysed using the Nikon-Elements AR Analysis software.

Cell proliferation assay

This assay was performed with the Cell Counting Kit 8(CCK-8) (Dojindo) according to the manufacturer's protocol. Briefly, 2000 cells were seeded into 96-well plates per well and cultured for 0h, 24h, 48h and 72h. Then, each well was added with 10 μl of CCK-8 solution and incubated 2 h. The absorbance value was determined at a wavelength of 450 nm using a microplate reader (BioTek). All experiments were performed in quintuplicate.

Colony-formation assay

500 cells per well were seeded on 6-well culture plates and cultured for two weeks. Then, the cells were fixed with 4% paraformaldehyde, and colonies were stained with 0.05% crystal violet and counted.

Cell migration assays

Cell invasion was determined using the 24-well chambers (Corning, #3422) with 8 μm pore polycarbonate membranes either uncoated (for migration) or coated with Matrigel Basement Membrane Matrix (for invasion; Corning). The chambers were rehydrated in serum-free medium. Complete medium with 20% FBS (600 μl) served as chemoattractant in the bottom chamber and 4 x 10^4 cells in serum-free medium were seeded with the top chambers and exposed to 21% O_2 or 1% O_2 at 37℃. After 24-48 h, cells on the top chambers were removed and the cells were stained with 0.05% crystal violet solution for 30 min and imaged on a bright-field microscope.

Supplementary Table S1. Primer sequences for quantitative RT-PCR, ChIP assay, mutant constructs, and shRNAs of AHNAK2.

quantitative RT-PCR Primers	
Gene	**Sequence**
AHNAK2-F	GAGAAGGAGGACACGGATGTTGC
AHNAK2-R	CCCCGCTTGCTCTTTATGGATTG
HIF1α-F	GAACGTCGAAAAGAAAAGTCTCG
HIF1α-R	CCTTATCAAGATGCGAACTCACA
HIF2α-F	GC TCTCCACGGCCTGATA
HIF2α-R	TTGTCACAC-CTATGG CATATCACC
ZO-1-F	ACCAGTAAGTCGTCCTGATCC
ZO-1-R	TCGGCCAAATCTTCTCACTCC
Vimentin-F	GACGCCATCAACACCGAGTT
Vimentin-R	CTTTGTCGTTGGTTAGCTGGT
OCT-4-F	CTTGAATCCCGAATGGAAAGGG
OCT-4-R	GTGTATATCCCAGGGTGATCCTC
Nanog-F	TTTGTGGGCCTGAAGAAAACT
Nanog-r	AGGGCTGTCCTGAATAAGCAG
Glut1-F	GGCCAAGAGTGTGCTAAAGAA
Glut1-R	ACAGCGTTGATGCCAGACAG
VEGFA-F	AGGGCAGAATCATCACGAAGT
VEGFA-R	AGGGTCTCGATTGGATGGCA
LDHA-F	ATGGCAACTCTAAAGGATCAGC
LDHA-R	CCAACCCCAACAACTGTAATCT
actin-F	CATGTACGTTGCTATCCAGGC
actin-r	CTCCTTAATGTCACGCACGAT
ACSS1-F	CACAGGACAGACAACAAGGTC
ACSS1-R	CCTGGGTATGGACGATGCC

ACLY -F	TCGGCCAAGGCAATTTCAGAG
ACLY -R	CGAGCATACTTGAACCGATTCT
ACC-F	ATGTCTGGCTTGCACCTAGTA
ACC-R	CCCCAAAGCGAGTAACAAATTCT
FASN-F	AAGGACCTGTCTAGGTTTGATGC
FASN-R	TGGCTTCATAGGTGACTTCCA
Lipin 1-F	CCAGCCCAATGGAAACCTCC
Lipin 1-R	AGGTGCATAGGGATAACTTCCTG
SCD1-F	TCTAGCTCCTATACCACCACCA
SCD1-R	TCGTCTCCAACTTATCTCCTCC
ELOVL6-F	AACGAGCAAAGTTTGAACTGAGG
ELOVL6-R	TCGAAGAGCACCGAATATACTGA
CHIP Primers	
Target	**Sequence**
HRE1-F	GCTAACGGGAACTGTCTAGG
HRE1-R	CTGCATGCATGAACACCCACAC
HRE2-F	TCTATGAGTGTGTGTGTCTGCC
HRE2-R	CATAGAGGCACACTCACACAC
HRE3-F	GTACCTGTGTATCTCTGTGT
HRE3-R	ACGGATACACACACACATAC
HRE4-F	TGCACTCTGATCCTCACCAA
HRE4-R	TGCCACTTAAGGCTCCAAGC
Mutant Construct Primers	
Target	**Sequence**
AP-MUT1-F	p-TGGGCCCGCTAGCTcacacacaTCTCCAAATGA
AP-MUT1-R	p-GGTGAGCCACCCGGGCACGATG
AP-MUT2-F	p-ATGAGCCCAGGAcacacacaGCCCCTGTGTGTAT
AP-MUT2-R	p-TTGGAGACCACGCACAGCTAGCGGG
AP-MUT3-F	p-TGTGTGTATGTATcacacacaATGGGTCTGTATCC

AP-MUT3-R	p-GGGGCACACGCATTCCTGGGCT
shRNA Sequence	
Target	**Sequence**
sh-AHNAK2 #1	TTGTTGTGTACACTCTAGCCTG
sh-AHNAK2 #2	TTGAACTTGCTGTCTTTGGTGG
sh-HIF1α#1	AGTTATGATTGTGAAGTTA
sh-HIF1α#2	GCGAAGTAAAGAATCTGAA
sh-HIF2α#2	GGAGACGGAGGTGTTCTAT
sh-HIF2α#3	GACAAGGTCTGCAAAGGGT

Supplementary Figures and Figure legends

Figure S1.

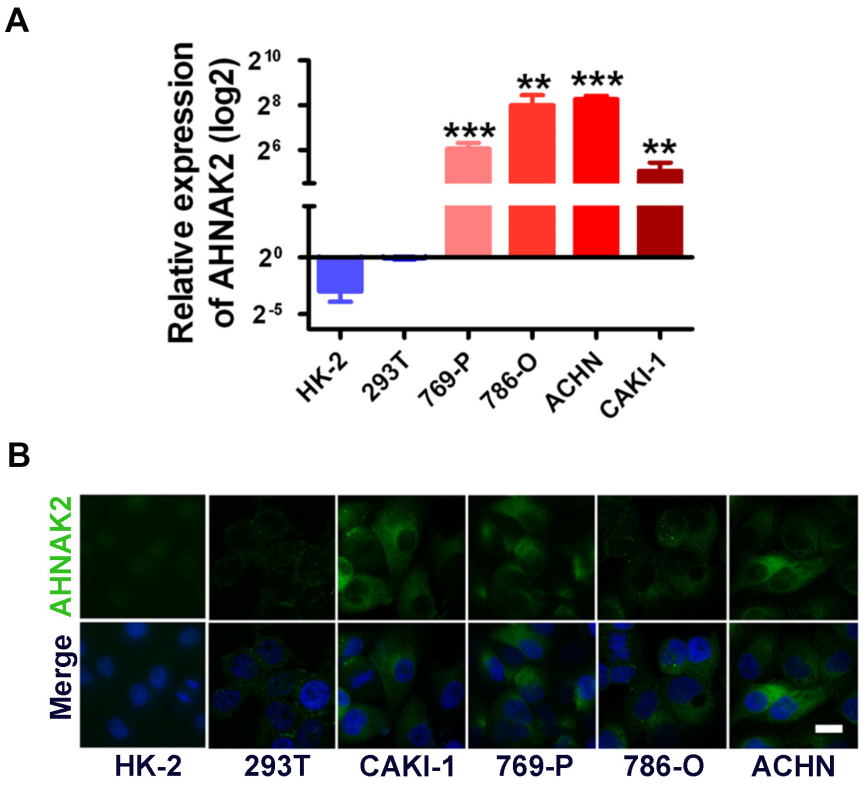

Figure S1. Expression of AHNAK2 in ccRCC cell lines.
(A) and **(B)** Q-PCR assay and immunofluorescence staining analysis of AHNAK2 transcription and expression in human RCC cell lines. Q-PCRs were normalized to the mRNA level of beta-actin. Scale bar = 20 μm. ** $p < 0.01$, *** $p < 0.001$. Data are mean ± SD of three independent experiments. Two-sided t test.

Figure S2.

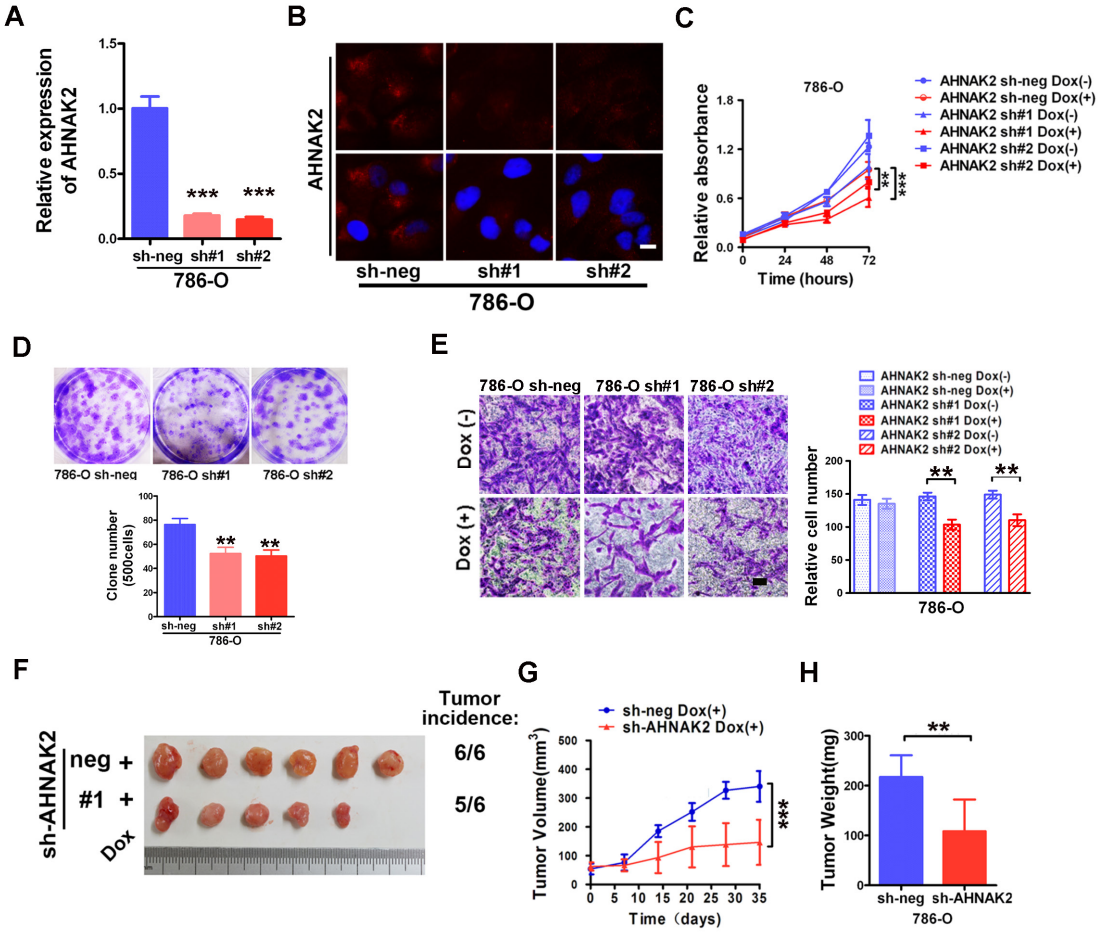

Figure S2. AHNAK2 knockdown inhibits the growth of ccRCC cells in vitro and in vivo.

(A) 786-O cells stably transfected with shRNAs were performed by qPCR analysis. **(B)** Representative immunofluorescence staining of AHNAK2 expression in each of the indicated cell lines. Scale bar = 20 μm. **(C)** The growth inhibition rates were measured in 786-O cells. **(D)** Representative images of clonogenic assays of 786-O cells stably expressing AHNAK2 shRNAs (sh#1 and #2) or control shRNA. **(E)** Representative images of migration assays of 786-O cells (left) and quantification of the relative migration cell numbers (right). Scale bar = 100 μm. **(F)** Representative images of xenografts derived from 786-O cells (n = 6/group). **(G)** Tumor volume and **(H)** weights of xenografts derived from control or AHNAK2-knockdowned 786-O cells were evaluated.

Figure S3.

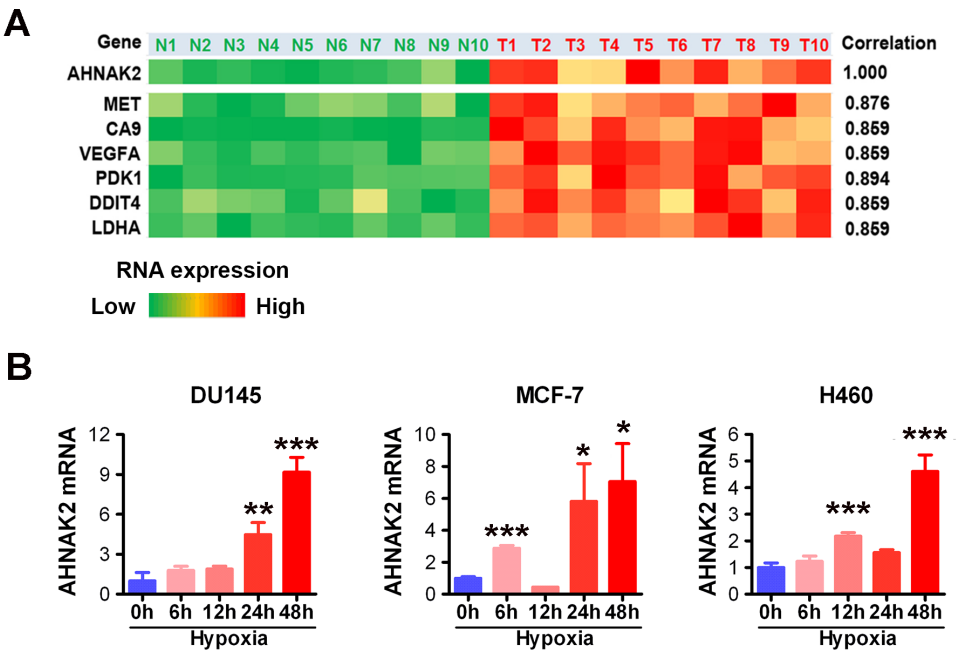

Figure S3. Upregulated expression of AHNAK2 induced by hypoxia in 3 different human cancer cell lines.

(A) Co-expression analysis of AHNAK2 in Grumz Renal dataset. **(B)** Q-PCR assay of AHNAK2 transcription in DU145, MCF-7 and H460 cells under hypoxia for 0, 6, 12, 24, and 48 hours respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S4

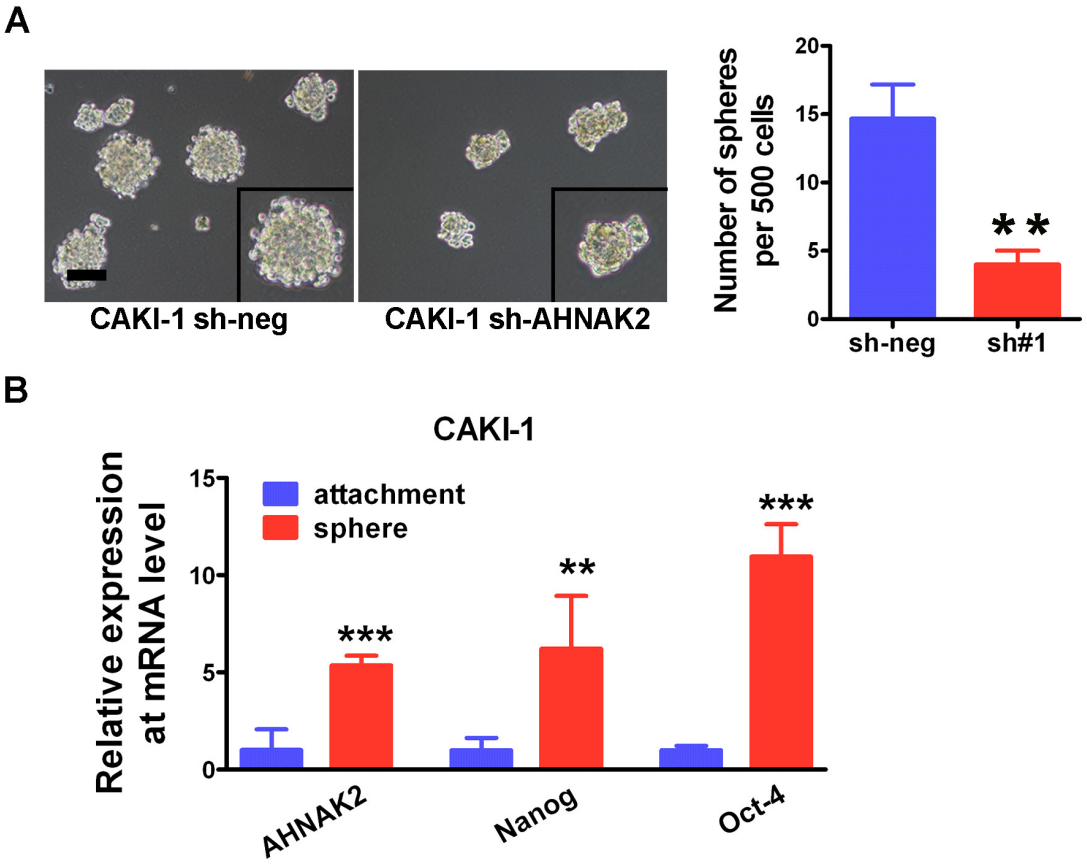

Figure S4. Effects of AHNAK2 expression on cancer stem cell-like properties.
(A) Representative images of spheres in CAKI-1 cells stably transfected with control sh-neg or AHNAK2 sh#1 (left) and quantification of the number of spheres (right). ** $p < 0.01$. **(B)** Relative expression of AHNAK2, Nanog and Oct-4 at the mRNA level in sphere cultures compared to attachment cultures of CAKI-1 cells . ** $p < 0.01$, *** $p < 0.001$.