Supplementary information

Supplementary Materials and Methods

Semi-quantitative RT-PCR and quantitative PCR. Semi-quantitative RT-PCR and quantitative PCR (qPCR) was performed as described previously (1). GAPDH was also amplified as an internal control. Primers used are listed in Suppl. Table S1.

Immunohistochemistry. The ChemMate EnVision Detection Kit (DAKO, Carpinteria, CA) was used for immunohistochemistry according to company's recommended procedure. JPH3 protein expression in tumor cells was scored based on the intensity of cytoplasmic staining using a 4-point system: 0, negative; 1, weakly positive; 2, positive and 3, strongly positive. To examine the association of JPH3 expression level with clinicopathological features, patients were divided into two groups: low JPH3 expression (0 and 1) or high JPH3 expression (2 and 3). Immunostaining was scored independently on separate occasions by two investigators who were blinded to the clinical information of patients, using JPH3 expression level in normal cells as a baseline.

Colony formation assays. For colony formation assays with monolayer culture, cells (2×10^5/well) were plated in a 12-well plate and transfected with JPH3-expression plasmid or empty vector (3μg each) using MegaTran 1.0 transfection reagent (Origene, Maryland, MD). Cells were collected and plated in a 10-cm dish 48 hours post-transfection, and selected for 16–20 days with G418 (0.6 mg/ml). Surviving colonies (≥50 cells/colony) were counted after Giemsa staining.

For soft agar assay, approximately ~500 stable transfected cells were suspended in medium containing 0.3% low-melt agarose, seeded into a six-well plate overlaid with 0.5% low-melt agarose, and allowed to grow for 2 weeks at 37℃ in 5% CO_2. Colonies containing more than 50 cells were counted under a microscope. Three wells were analyzed for each experiment.

Wound-healing assay. Cell motility was assessed using a scratch wound assay. Stably transfected cells were cultured in 35 mm dishes until confluent. A single scratch was produced in the cell layer using a sterile tip and washed twice with fresh medium. After incubation for 24 and 48 hours, the cells were photographed under a phase contrast microscope. The experiments were performed in triplicate.

Transwell cell migration assay. Cells were trypsinized and resuspended in corresponding medium containing 1% fetal bovine serum at a density of 1×10^6 cells/ml. Cell suspension (100 μl) was added into the upper chamber of a transwell plate (Corning, NY, U.S.) consisting of inserts containing PET membranes with 8-μm pores. Medium (600 μl) containing 10% fetal bovine serum was placed in the lower chamber. After 48-hour incubation at 37℃, cells remaining in the upper chamber were removed carefully with a cotton swab and the membrane was removed using operating surgical knife. The side facing the lower chamber was stained with 0.05% crystal violet and the attached cells were counted under a light microscope. The experiment was performed in triplicate.

Apoptosis assay. Percentage of apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit I (BD Clontech, Palo Alto, CA) with flow cytometry according

to the manufacturer's instruction.

Western blot analysis. Cells were harvested from cultured dishes and lysed in lysis buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 140 mM NaCl, 1% NP-40, 1% aprotinin, 1 mM phenylemethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate). Protein concentration was determined using a BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL, U.S.). Cell lysates (40 µg protein/line) were separated on a 5 to 20% SDS-PAGE gel for nitrocellulose membrane blotting. Blotted membranes were blocked with 5% skim milk for 1 hour and incubated with primary antibodies which are listed in Suppl. Table S2, respectively. Detection was carried out using the ECL kit (Pierce Chemical Co., Rockford, IL) and the blots were developed using a Fujifilm Las-4000 Imaging System.

AIF and p53 immunofluorescence. Cells grown on coverslips were fixed in methanol, incubated with an anti-AIF or an anti-p53 antibody, and detected by anti-rabbit immunoglobulin G (IgG) conjugated with Alexa Fluor 594 (Invitrogen). Nuclei were stained with DAPI, and the red and blue fluorescence was observed with a Fluorescence microscope (Olympus BX51).

Supplementary Table 1. PCR primers used in the study

Primers	Sequences (5'-3')	Product size (bp)
For JPH3 RT-PCR		
JPH3-F	AATCCTTGCCTGTCGCTCTA	v1 and v4: 292
JPH3-R	CCCAATCGTGTGGTTCTTCT	
JPH3-F2	AAT ACG AAGGGACCTGGAGC	v2: 296
JPH3-R2	GTTTGTTTGGGGTCTGGCTT	v3: 442
For detecting methylated JPH3 promoter by MSP		
JPH3-m3	AGACGTTGGTTAGGTTTCGC	119
JPH3-m4	CGCGACCCCGAAACGCG	
For detecting unmethylated JPH3 promoter by MSP		
JPH3-u3	GAGATGTTGGTTAGGTTTTGT	124
JPH3-u4	AAAACACAACCCCAAAACACA	
For BGS analysis of JPH3 methylation		
JPH3-BGS1	GGTTTTTAATATGGTGTAGT	554
JPH3-BGS2	AAATTAAACCTACCCCCACTAA	
Control for RT-PCR		
GAPDH-F	TCCTGTGGCATCCACGAAACT	496
GAPDH-R	GAAGCATTTGCGGTGGACGAT	
Control for qPCR		
HPRT1-qPCR-F	AGACTTTGCTTTCCTTGGTCAG	151
HPRT1-qPCR-R	TCAAGGGCATATCCTACAACAA	

Supplementary Table 2. Antibodies used in the paper

Antibody	Catalogue number	Company
TRB3	sc-271572	Santa Cruz
CHOP	2895	Cell Signaling Technology
Bip	3177	Cell Signaling Technology
IRE1a	3294	Cell Signaling Technology
Ero1-La	3264	Cell Signaling Technology
PERK	5683	Cell Signaling Technology
Caspase 3	BS7004	Bioworld
Caspase 9	sc-8355	Santa Cruz
Cleaved PARP	556494	BD
Caspase 4	4450	Cell Signaling Technology
Caspase 7	12827	Cell Signaling Technology
Caspase 8	9746	Cell Signaling Technology
Cyto C	556433	BD
Smac	2409	ProSci
AIF	5318	Cell Signaling Technology
Bcl2	2870	Cell Signaling Technology
p-Bcl2 (Ser70)	2827	Cell Signaling Technology
Mcl-1	5453	Cell Signaling Technology
Bcl-XL	2764	Cell Signaling Technology
Bid	2002	Cell Signaling Technology
Bad	9237	Cell Signaling Technology
Bax	5023	Cell Signaling Technology
Bak	12105	Cell Signaling Technology
Pan-Akt	AA326	Beyotime
p-Akt (Ser473)	AA329	Beyotime
p-JNK	AJ516	Beyotime
p-p38	AM063	Beyotime
p-p44/42	AM071	Beyotime
p53	sc-126	Santa Cruz
GAPDH	2118	Cell Signaling Technology
Tubulin	2144	Cell Signaling Technology

Supplementary figure legends

Supplementary figure 1. (A) Structure of the *JPH3* gene and reported transcript splices. Expression of *JPH3* transcript variants and *JPH4* in (B) CRC and gastric tumor cell lines and (C) primary tumors, measured by semi-quantitative RT-PCR. CRC, colorectal cancer; GsCa, gastric cancer.

Supplementary figure 2. (A) Gene location of *JPH3* on Chr 16. (B) The sequence of *JPH3* promoter and exon 1 is indicated (-529 bp to +44 bp). The 81 CpG sites sequenced in *JPH3* promoter and exon 1 are highlighted in bold. The positions of forward and reverse primers used for BGS and MSP analyses are underlined. The transcription initiation site is indicated by a bent arrow. The translation start site (ATG) is boxed. The MSP and BGS primers with green names underlined are used in the study.

Supplementary figure 3. Endogenous and ectopic expression of *JPH3* in colorectal and gastric cell lines as measured by qPCR. CRC, colorectal cancer.

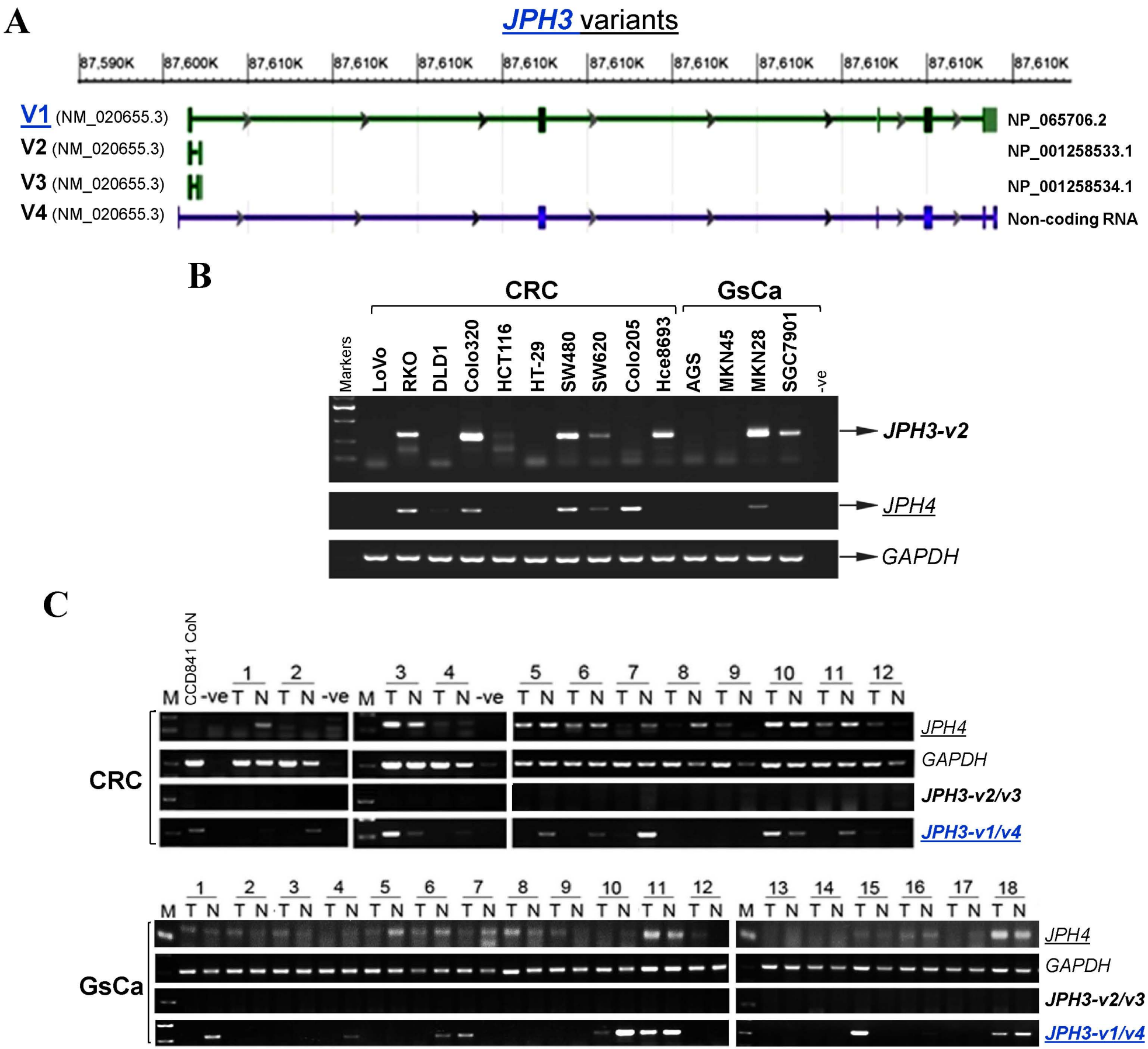
Supplementary figure 4. Ectopic JPH3 expression had no apparent effect on p53 translocation. Expression shown by immunofluorescence and Western blot in (A) HCT116 and (B) MKN-28 cells.

Reference

1. Hu X, Sui X, Li L, Huang X, Rong R, Su X, Shi Q, Mo L, Shu X, Kuang Y, et al. Protocadherin 17 acts as a tumour suppressor inducing tumour cell apoptosis

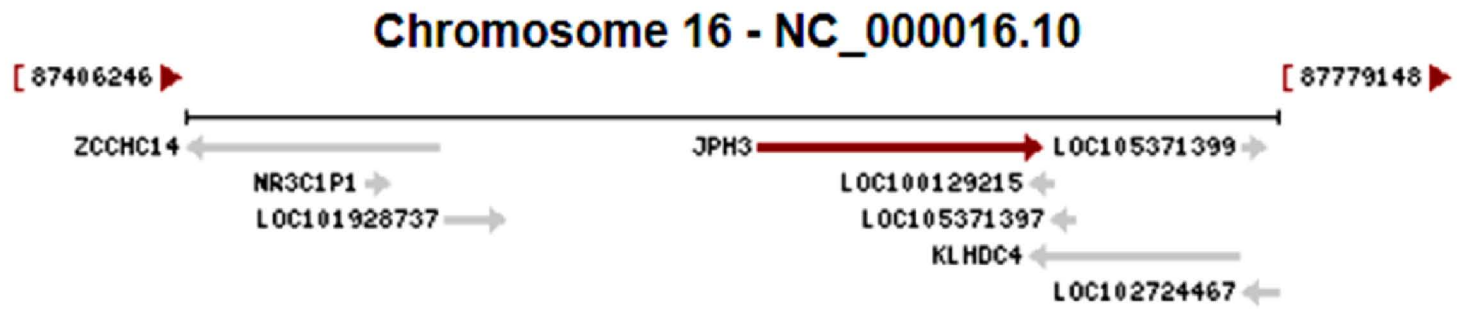
and autophagy, and is frequently methylated in gastric and colorectal cancers. *J Pathol.* 2013;229(1):62-73.

Suppl fig1


A
JPH3 variants
87,590K
87,600K
87,610K
V1 (NM_020655.3)
V2 (NM_020655.3)
V3 (NM_020655.3)
V4 (NM_020655.3)
NP_065706.2
NP_001258533.1
NP_001258534.1
Non-coding RNA
B
CRC
GsCa
Markers
LoVo
RKO
DLD1
Colo320
HCT116
HT-29
SW480
SW620
Colo205
Hce8693
AGS
MKN45
MKN28
SGC7901
-ve
JPH3-v2
JPH4
GAPDH
C
CCD841 CoN
M
-ve
T
N
CRC
GsCa
JPH4
GAPDH
JPH3-v2/v3
JPH3-v1/v4

Suppl fig2

A



B

GAGCCGCCGCATTGCTGCTGCTGCTGCCGCCGCCGCCCCGCGCCCGGCCGC -529

GGCCCCCAATATGGTGCAGCCGCCAGCGCCGCCGCCCGTGCCGCCGCCGCG -477

BGS1 m5

CCCGCGGGCCCCGCCGCCGCCCTCGGGCGCCCGCAGCGCGGCAGCCGCAGGT -425

GGGGGGCCGCGGCCCGGGCCTGAGCTGCCCCCCGCCCGGCCTCGGCGCGGCG -373

m11

CCCTCCCCGCGGGGCTGCCGCGCGAGGGGCCCTCTCGCCGCTGAGGTGGAGCC -321

m6

GCGGGCCCCGCCGCCGCCGCCTCCTGCCCCCTCCCTCCCTCCTCCCCCTCCCCC -269

m22

TCCCCTCCGGTCCTGTCTCCAGCGGGAGCGCGAGACGCTGGTCAGGCTCCGC -217

m3

GGCGCAGCTCGAAAAGGAATAATCGCCCCGATTGACTGAAATTCCTCCGGA -165

GCCGGCGCCGCGCCGCCCGCGCCCGAGACCGCGCTCCGGGGCCGCGTCCTC -113

m4

CTCTCCTCCGGAAAACGCTCGCGACCCAGGGCCGCCGGCGGCCGCGACTCTG -61

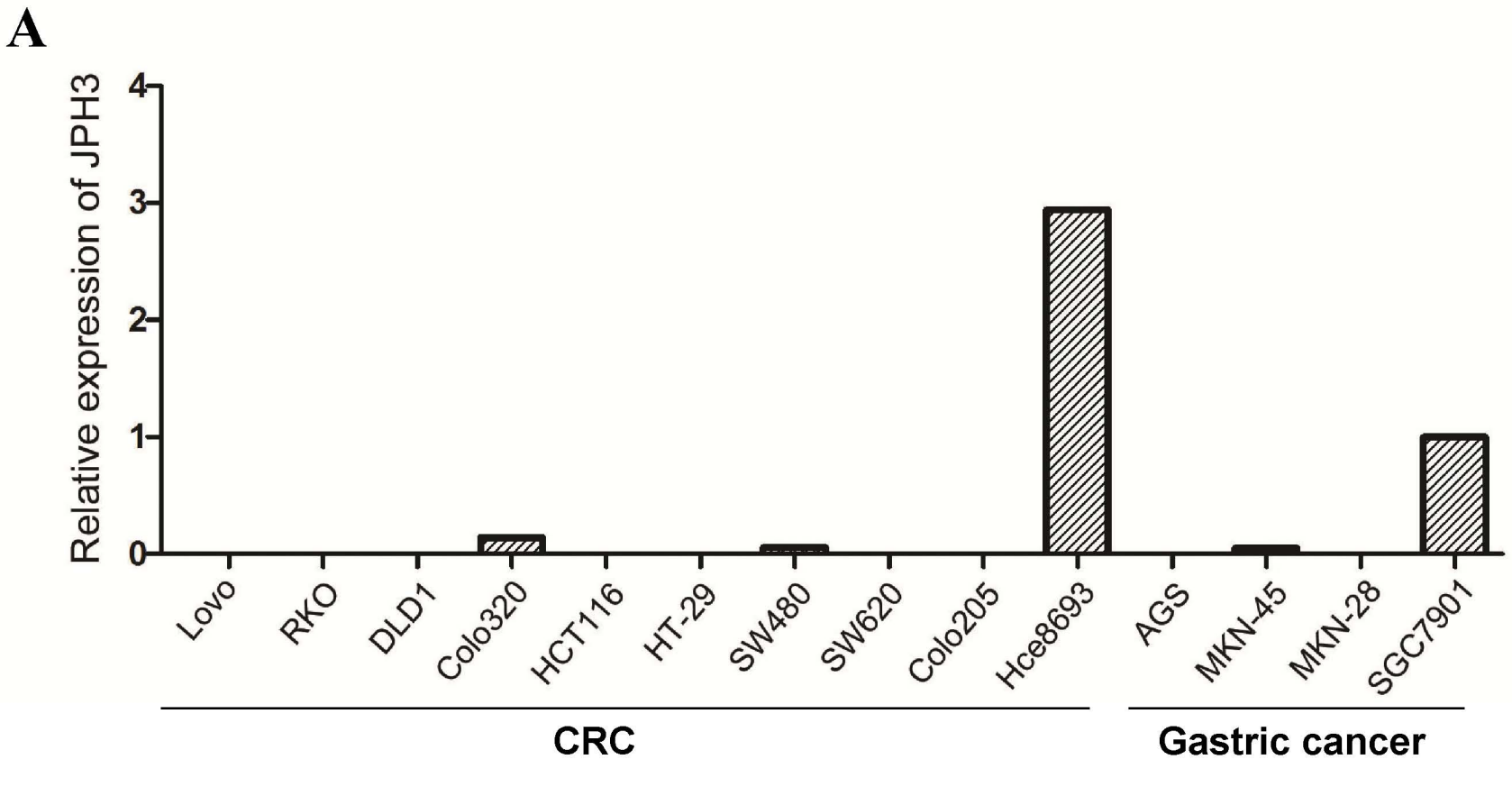
CTGTGTCGATCGCCTGAGTCCGTTTTCACCGTTTGCGGGATCTGGAACCGAG -9

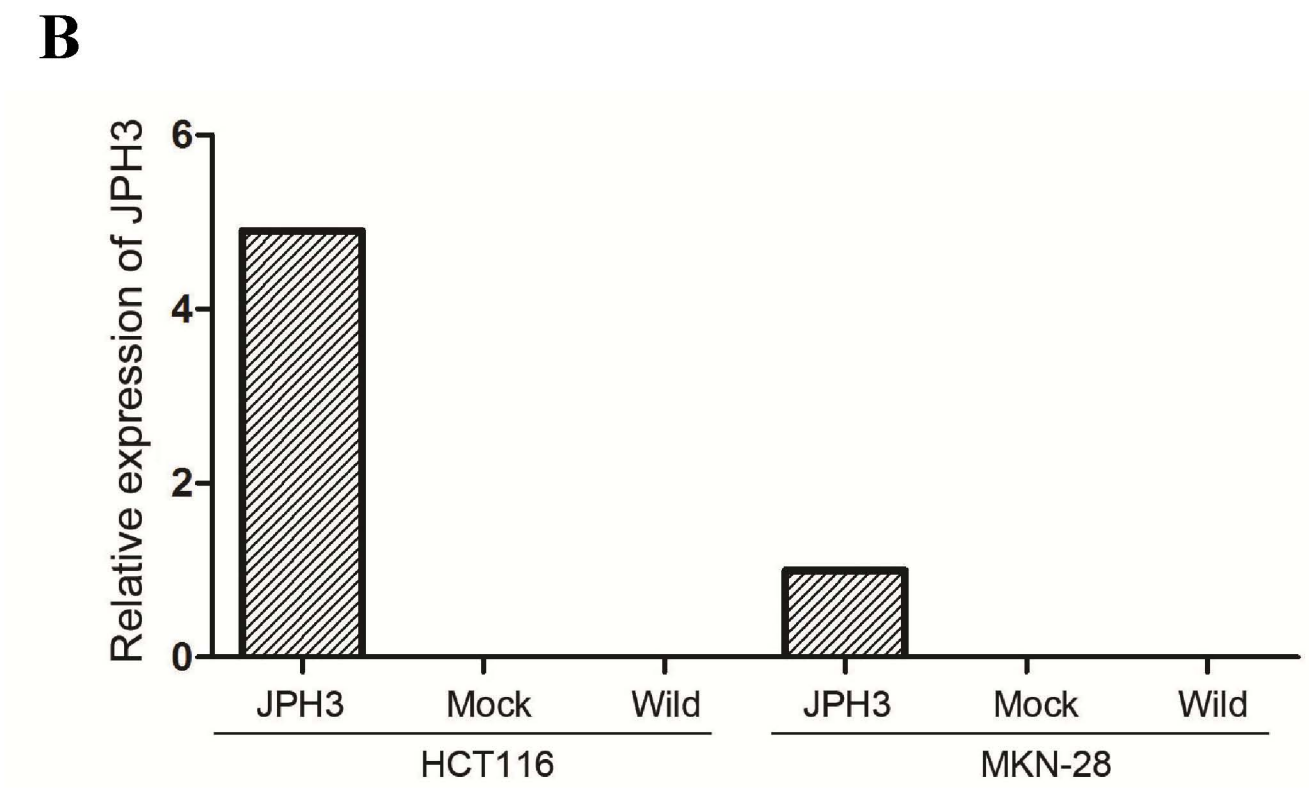
exon 1

TTACATGCATGTCCAGTGGGGGCAGGTTTAATTTTGACGACGGAGGGTCCTA + 44

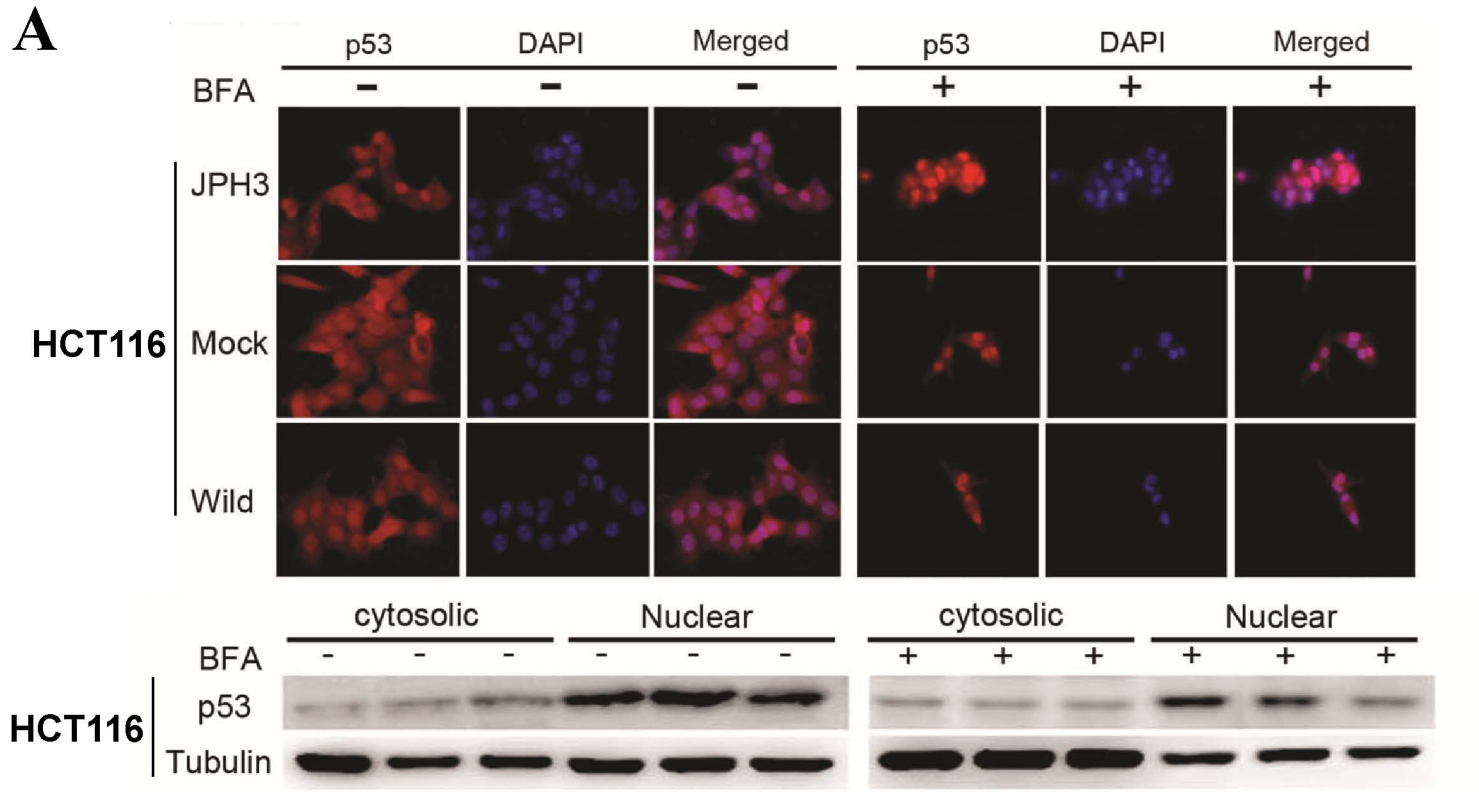
BGS2

Suppl fig3


A
Relative expression of JPH3
0
1
2
3
4
Lovo
RKO
DLD1
Colo320
HCT116
HT-29
SW480
SW620
Colo205
Hce8693
AGS
MKN-45
MKN-28
SGC7901
CRC
Gastric cancer


B
Relative expression of JPH3
0
2
4
6
JPH3
Mock
Wild
JPH3
Mock
Wild
HCT116
MKN-28

Suppl fig4


A
p53
DAPI
Merged
p53
DAPI
Merged
BFA
-
-
-
+
+
+
JPH3
HCT116
Mock
Wild
cytosolic
Nuclear
cytosolic
Nuclear
BFA
- - - - - -
+ + + + + +
HCT116
p53
Tubulin
B
p53
DAPI
Merged
p53
DAPI
Merged
BFA
-
-
-
+
+
+
JPH3
MKN-28
Mock
Wild
cytosolic
Nuclear
cytosolic
Nuclear
BFA
- - - - - -
+ + + + + +
MKN-28
p53
Tubulin