Supplementary Materials and Methods

Clinical specimens

Human pancreatic cancer tissues and paired adjacent normal tissues were collected from 96 pancreatic cancer patients at the Second Affiliated Hospital of Nanchang University. Informed consent was obtained from all patients with approval by the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University.

Quantitative RT-PCR

Total RNA of PC cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR for mRNA detection was performed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, RR047A) and TB GREEN Premix Ex Taq (TaKaRa, RR420A). Relative quantification of the mRNA levels was performed using the comparative Ct method, and Tubulin was used as the reference gene for mRNA expression, each experiment was run in triplicate and a mean value was used for the comparison of each group. Primer sequences are listed in below.

Gene	Primer Sequences
TRIM16	forward: CTTGCAGCCGCATCAGGTGA
	reverse: GGTGCACTGGAGTTCAGCCT
SIX1	forward: CGATGCTGCCGTCGTTTGGC
	reverse: TGGTTGTGAGGCGAGAACTG
NIK	forward: CCAGCTGCCATCTCTATCATC
	reverse: AAACAGAGTTGCCCAAGGCC
GLUT1	forward: TTGGCTCCGGTATCGTCAAC
	reverse: GGCCACGATGCTCAGATAGG
HK2	forward: CAGCACAAAGCAGTCGGACC
	reverse: CAGAGAGGCGCATGTGGTAG
PGK1	forward: CTGTCATCCTGCTGGAGA
	reverse: GCCTTCTGTGGCAGATTGAC
LDHA	forward: CATGGCCTGTGCCATCAGTA
	reverse: AGATATCCACTTTGCCAGAGACA
Tubulin	forward: GATGTATGCCAAGCGTGCCT
	reverse: AGACCACAACTTTCTGTGTTGG

Immunohistochemistry (IHC)

Pancreatic cancer and adjacent normal tissues were fixed with formalin, then, dehydration with xylene and graded alcohol and embedded in paraffin. A slide was subjected to antigen retrieval in 0.01 M citrate buffer, blocked in goat serum for 30 min and incubated with antibody overnight at 4°C in optimal dilution, then, slides were further incubated with biotin-conjugated secondary antibody for 30 minutes in 37°C and developed by 3,3-diaminobenzidine(DAB) following by hematoxylin staining. The stained IHC slides were scanned and the scores are ranged from 0-12.

Western blot

For western blot analysis, equal amounts of protein were isolated and fractionated via sodium dodecyl sulfate-polyacryla-mide gelelectrophoresis (SDS-PAGE), the separated proteins were transferred to PVDF membranes, blocked with 5% nonfat milk. The membranes were then incubated with primary antibodies according to the recommended concentration at 4°C overnight, followed by HRP-conjugated secondary antibodies, washed three times with 1 × TBST, then, The results were obtained via chemiluminescence using Quantity-One software (Bio-Rad, Hercules, CA, USA).

Primary antibodies are listed in below.

Antibodies	SOURCE	IDENTIFIER
TRIM16	Abcam	Cat# ab251749
TRIM16	Abcam	Cat# ab72129
SIX1	proteintech	Cat# 10709-1-AP
NIK	Abcam	Cat# ab203568
GLUT1	Abcam	Cat# ab115730
HK2	proteintech	Cat# 22029-1-AP
PGK1	proteintech	Cat# 17811-1-AP
LDHA	proteintech	Cat# 19987-1-AP
Tubulin	proteintech	Cat# 66031-1-Ig
Ubiquitin	Abcam	Cat# ab134953
Ubiquitin (linkage-specific K48)	Abcam	Cat# ab140601
Ubiquitin (linkage-specific K63)	Abcam	Cat# ab179434
GST	proteintech	Cat# 66001-2-Ig
His	proteintech	Cat# 66005-1-Ig
Flag	proteintech	Cat# 20543-1-AP

Cell culture

The human pancreatic cancer cell lines PANC-1, BxPC-3, AsPC-1, CaPan-1, CFPAC-1, normal pancreatic duct epithelial cell HPDE6-C7 and HEK293T were purchased from the Shanghai Institute of Cell Biology, China. All cells were cultured in the recommended DMEM (Gibco) supplemented with 10% fetal bovine serum and were exposed to 100 U/mL penicillin and streptomycin at 37°C in 5% CO_2.

The plasmids construction

The short hairpin RNAs for TRIM16, SIX1 and NIK silencing were synthesized by GeneChem, Company (Shanghai, China). A TRIM16 (NM_006470.4), SIX1 (NM_005982.4) or NIK (NM_003954.5) overexpression plasmids was constructed by introducing the respective cDNA in 6*His tagged-pcDNA3.1(+), HA tagged-pcDNA3.1(+) and Flag tagged-pcDNA3.1(+) (GeneChem, Shanghai, China). All shRNA and overexpression plasmids were embedded into pGPU6/GFP/Neo vector lentivirus.

Plasmids expressing fusion protein were constructed by introducing the respective cDNA in GST tagged-pGEX-4T-1, 6*His tagged-pGEX-4T-1 and Flag tagged-pGEX-4T-1 (GeneChem, Shanghai, China). All the plasmids were transfected into BL21 (DE3) Escherichia coli cells, and the fusion protein was expressed under suitable temperature and IPTG induction. Using ÄKTA™pure (GE Healthcare, America) purified the fusion protein.

Lentivirus transfection and generation of stable cells

The day before transfection, 1×10^5 cells were seeded into 6-well plates. Cells were seeded at 70% confluence 12 hours before infection, and the cell culture medium was replaced with medium containing lentivirus. After infection for 12 hours, the medium was replaced with fresh medium, and 48 h later the infected cells were selected with 5 μg/ml puromycin (InvivoGen, San Diego, CA, USA). To select for stably transfected cells, cells were cultured in high-glucose DMEM with 5 μg/mL puromycin for 4 weeks. Clones demonstrating puromycin resistance and expressing GFP were selected and expanded. Western blotting analysis was used to confirm the effect of the constructed stable cells.

In vitro invasion and migration assays

The pancreatic cancer cells' migration abilities were detected in a Transwell Boyden Chamber (pore size of 8 μm, BD Biosciences). Briefly, 1×10^5 pancreatic cancer cells were resuspended in FBS-free culture

medium and then added into the upper Boyden chamber, while the lower chamber was filled with culture medium containing 10% FBS. After 24 h, cells in the upper chamber that had passed through the polycarbonate membrane between the upper and lower compartment and adhered to its lower side were stained with crystal violet. The cells were counted, and we took images of them. For the cell invasion assay, the polycarbonate membranes were precoated with a matrix gel.

In vivo metastasis assay

The nude mice (male BALB/c-nu/nu, 4-6 weeks old) were purchased from the Animal Center of Nanjing Medical University. 5×10^5 pancreatic cancer cells were stably transduced with firefly luciferase gene and injected into the tail vein of BALB/c nude mice. After 6-8 weeks, for in vivo signal detection, the mice were anesthetized with isofluorane and then imaged in a Lumina Series III IVIS instrument (PerkinElmer, USA). Then, the mice were euthanized, and their livers were removed and stained with haematoxylin and eosin stain (H&E) for pathological examination. The animal work was approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University.

Glucose uptake assays, lactic acid production assays, ATP production rate and extracellular acidification rate (ECAR) assays

These metabolic profiles were tested by using the Glucose Uptake Assay Kit (Abnova Corporation, Taipei City, Taiwan), Lactate (human) ELISA Kit (BioVision, San Francisco, USA), Agilent Seahorse XF Real-Time ATP Rate Assay Kit and Seahorse XF Glycolysis Stress Test kit (Agilent, California, USA). These kits were used according to the manufacturer's instructions.

Isobaric tags for relative and absolute quantitation (iTRAQ)

Firstly, we transfected shRNA targeting TRIM16 into pancreatic cancer cells. The shRNA sequence is CACACCAUAGUCUCCCUGGTT. Then, cells were collected and extracting the proteins using extraction buffer (50 mM phosphate-buffered saline, 100 mM NaCl, 1 mM PMSF, and 1 mM EDTA). Groups shNC-1, shNC-2 and shNC-3 (shNC = controls) and groups shTRIM16-1, shTRIM16-2 and shTRIM16-3 were formed. After precipitated with acetone, the protein samples were dissolved in TEAB buffer, and then reduced, alkylated, trypsin-digested, and labeled according to the manufacturer's instructions. After the combination of six labeled samples, the iTRAQ-labeled peptides were fractionated by high-performance liquid chromatography (HPLC), and analyzed by high-resolution Orbitrap Fusion mass spectrometer. The mass spectrometer raw data were analyzed with Proteome Discoverer Software version 2.1 (Thermo Fisher Scientific).

In vivo ubiquitination assay

Pancreatic cancer cells were exposed to MG132 (15 mmol/L) for 12 h, and then the cell lysate was immunoprecipitated with primary antibody. The ubiquitination of protein was detected by an anti-ubiquitin antibody.

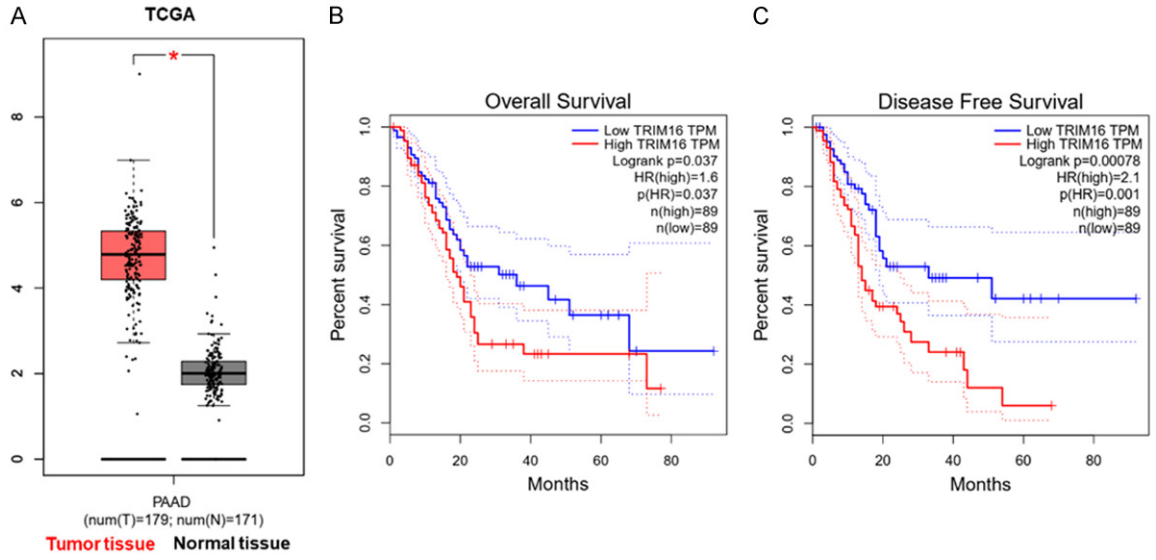
Co-immunoprecipitation (co-IP) and GST pull-down assay

For co-immunoprecipitation, cells were harvested in western and IP lysis buffer, rotated in rotator at 4°C for 2 h, centrifuged at 10,000 rpm for 10 min to get rid of cellular debris, then, the supernatant was incubated with primary antibody at 4°C for 2 h and mixed with proteinA/GPLUS-Agarose overnight, the immuno-complexes and collected and washed five times by western and IP lysis buffer after centrifugation, the pellet was mixed with SDS-PAGE sample buffer and boiling for 10 min following by western blot and autoradiography.

GST-fusion protein or control GST was added into the cell lysates harvested from the cells transfected with 6*His-tagged SIX1 or Flag-tagged NIK. After being incubated with Glutathione beads (Sigma-Aldrich, St. Louis, MO, USA) for 2 h, the bound proteins were subjected to western blot analysis using His or Flag antibodies.

Surface plasmon resonance (SPR) analysis

The binding affinity of NIK protein to TRAF3 protein was detected with or without TRIM16 by using a BIAcore X100 system to perform the SPR analysis. Purified NIK protein was covalently coupled to a CM5 sensor chip (GE Healthcare). For kinetic measurements, a series of concentration of TRAF3 + vehicle or TRAF3 + TRIM16 proteins (2000 nM, 1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) were flowed over the NIK chip surface. The binding kinetics were analyzed using the 1:1 Langmuir binding model (Biacore Evaluation X100 software, version 2.0). The apparent equilibrium dissociation constant (KD) was calculated as the ratio Koff/Kon (Kon: the association rates, Koff: the dissociation rates). The higher the KD value, the lower the binding affinity.



Supplementary Figure 1. A. The mRNA level of TRIM16 was upregulated in pancreatic cancer tissues via the TCGA dataset. B and C. Kaplan-Meier plots representing probabilities of overall survival and disease-free survival in the TCGA dataset according to expression level of TRIM16.

Supplementary Table 1. Correlation between TRIM16 and clinicopathologic characteristics of 96 pancreatic cancer patients

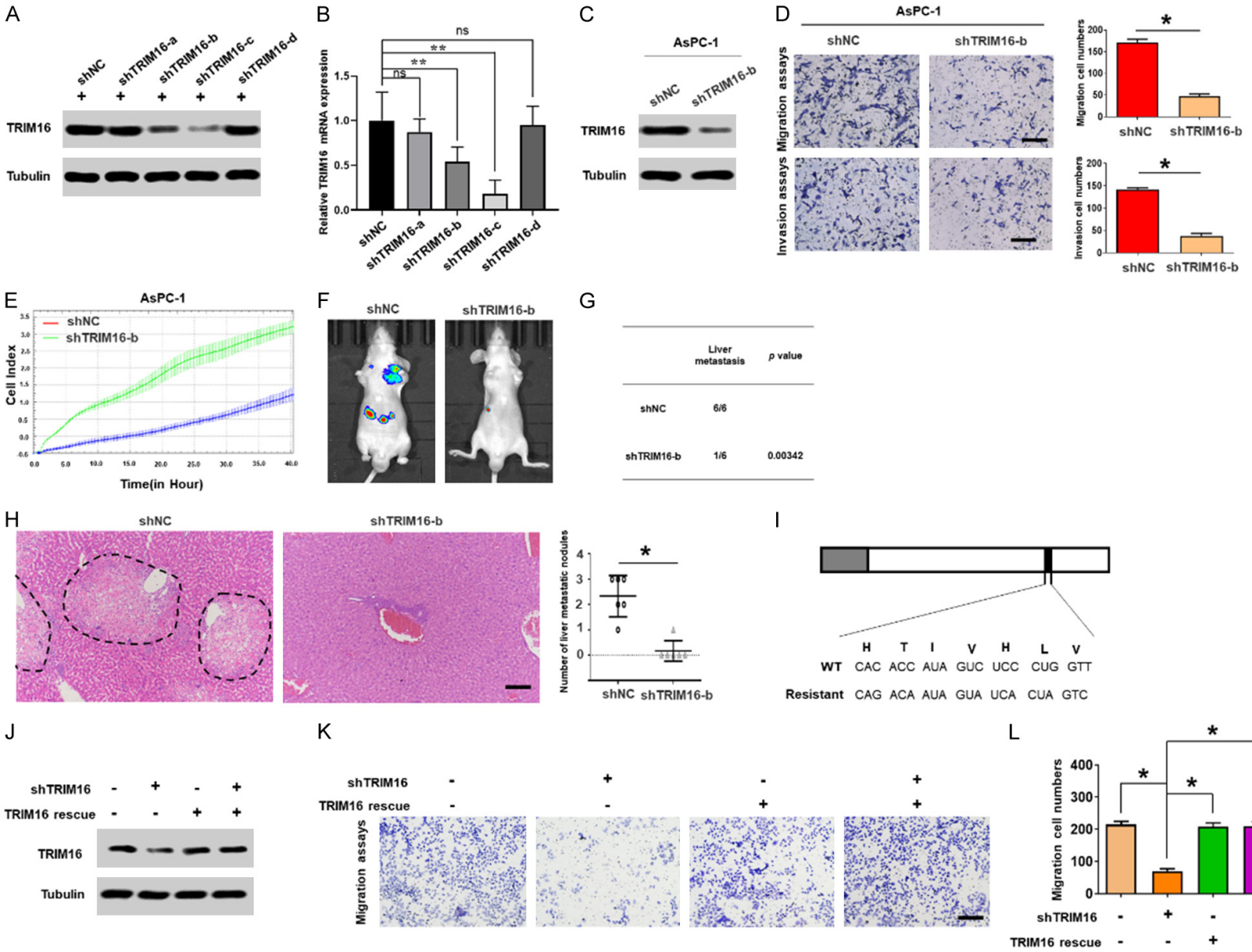
Variables	clinicopathological characteristics	numbers	TRIM16 high expression	TRIM16 low expression	*p* value
Age					0.749
	<60	42	27	15	
	≥60	54	33	21	
Gender					0.711
	Female	45	29	16	
	Male	51	31	20	
Tumor site					0.236
	Pancreatic head	58	39	19	
	Pancreatic body and tail	38	21	17	
Tumor size					0.114
	≤4 cm	46	25	21	
	>4 cm	50	35	15	
Vessel invasion					<0.001***
	NO	43	18	25	
	YES	53	42	11	
Lymph node metastasis					<0.001***
	Negative	37	14	23	
	Positive	59	46	13	
TNM stage					0.001**
	I-II	41	18	23	
	III-IV	55	42	13	
CEA					0.893
	Normal	18	11	7	
	Abnormal	78	49	29	

**P<0.01, ***P<0.001.

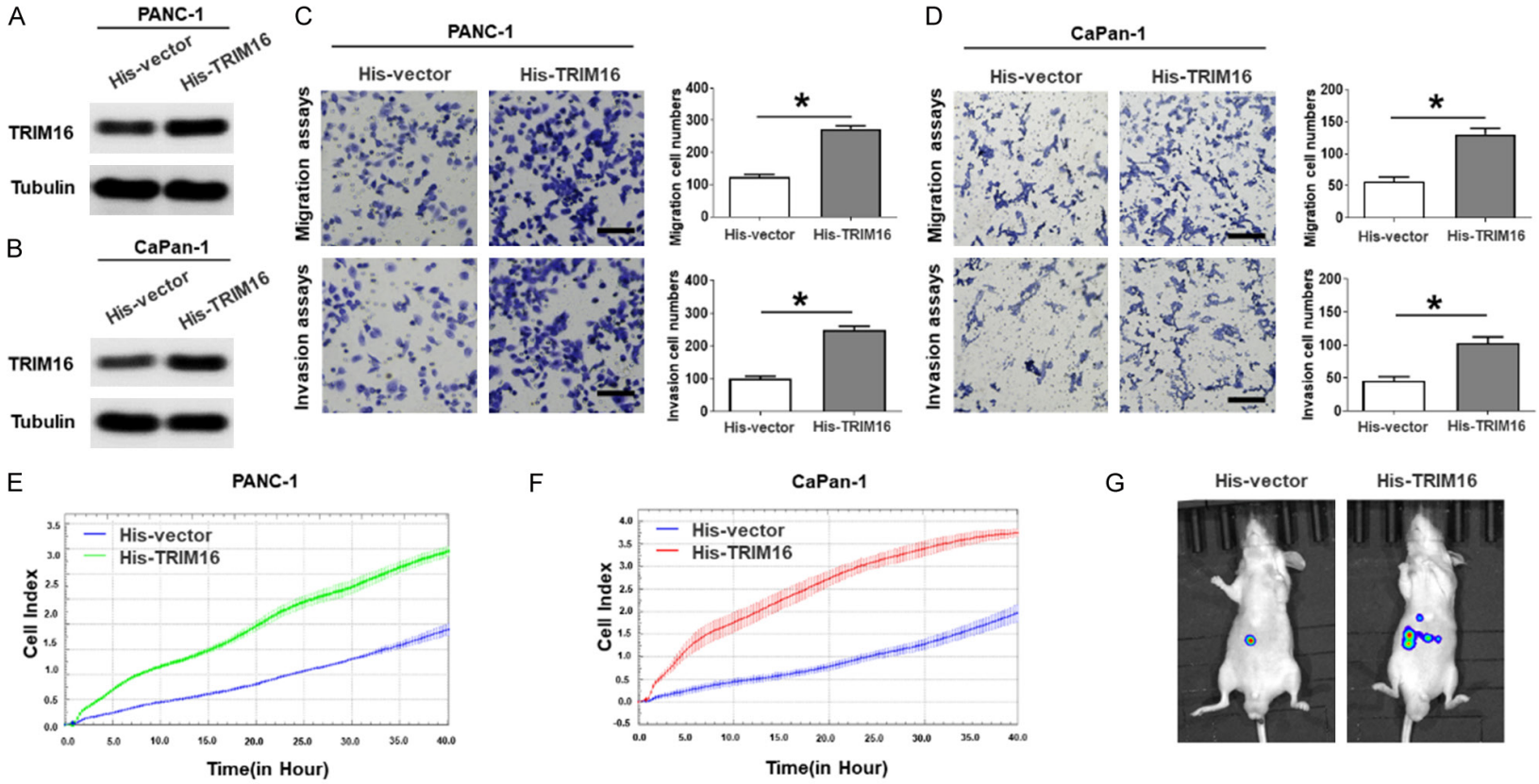
Supplementary Table 2. Univariate and multivariate analyses of overall survival in pancreatic cancer patients

Parameters	Univariate			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (≥60 vs <60)	1.165	0.723-3.261	0.653	-	-	-
Gender (Female vs Male)	1.576	0.523-2.341	0.686	-	-	-
Tumor site (Head vs Body and tail)	1.076	0.943-3.655	0.136	-	-	-
Vessel invasion (YES vs NO)	2.768	1.665-4.132	0.025*	2.124	1.416-3.172	0.012*
Lymph node metastasis (Positive vs Negative)	1.673	1.184-2.262	0.029*	1.394	0.893-1.926	0.095
TNM stage (III-IV vs I-II)	2.653	2.132-3.243	0.016*	2.214	1.696-2.975	0.017*
TRIM16 expression (High vs Low)	3.174	1.952-5.128	0.007**	2.597	1.611-4.332	0.019*

HR, Hazard Ratio; CI, Confidence Interval; *P<0.05; **P<0.01.


A
shNC
shTRIM16-a
shTRIM16-b
shTRIM16-c
shTRIM16-d
TRIM16
Tubulin
B
Relative TRIM16 mRNA expression
ns
**
**
ns
C
AsPC-1
shNC
shTRIM16-b
TRIM16
Tubulin
D
AsPC-1
shNC
shTRIM16-b
Migration assays
Invasion assays
Migration cell numbers
Invasion cell numbers
E
AsPC-1
shNC
shTRIM16-b
Cell Index
Time(in Hour)
F
shNC
shTRIM16-b
G
Liver metastasis
p value
shNC 6/6
shTRIM16-b 1/6 0.00342
H
shNC
shTRIM16-b
Number of liver metastatic nodules
I
H T I V H L V
WT CAC ACC AUA GUC UCC CUG GTT
Resistant CAG ACA AUA GUA UCA CUA GTC
J
shTRIM16
TRIM16 rescue
TRIM16
Tubulin
K
shTRIM16
TRIM16 rescue
Migration assays
L
Migration cell numbers
shTRIM16
TRIM16 rescue

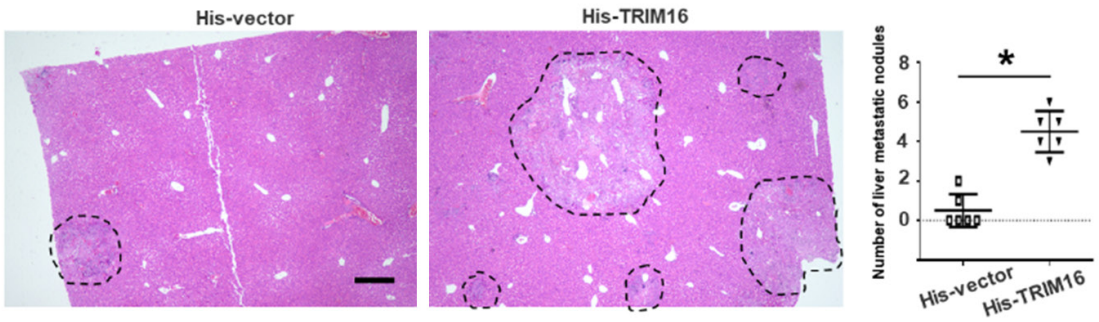
Supplementary Figure 2. ShRNA-resistant TRIM16 plasmid rescued the expression of TRIM16 and recovered cell's migration in shTRIM16-pancreatic cancer cells. A and B. Western blotting and qRT-PCR analyses were used to detect the expression level of TRIM16, Tubulin was used as a loading control. C. Western blotting analyses were used to detect the expression level of TRIM16, Tubulin was used as a loading control. D. Transwell migration and transwell invasion assays were used to detect the cells' migration and invasion abilities. The image was captured at 400 × magnification. Scale bar, 50 μm. *P<0.05. E. RTCA assays were performed to detect the cells' metastasis ability. F. AsPC-1 cells transfected with TRIM16-b were injected into the tail vein of nude mice, and the in vivo liver metastatic signal detection were imaged by a Lumina Series III IVIS instrument. G. The incidence of liver metastasis were measured after 6-8 weeks. n = 6, **P<0.01. H. Representative image (left; magnification: × 100) and quantification (right) of H&E staining of liver metastatic nodules. n = 6. Scale bar, 50 μm. *P<0.05. I. Construction of shRNA-resistant TRIM16 plasmid. J. Western blotting analyses were used to detect the expression level of TRIM16, Tubulin was used as a loading control. K. Transwell migration assays were used to detect the cells' migration abilities. The image was captured at 400 × magnification. Scale bar, 50 μm. *P<0.05. L. Quantification of migrated cells in different groups. *P<0.05.



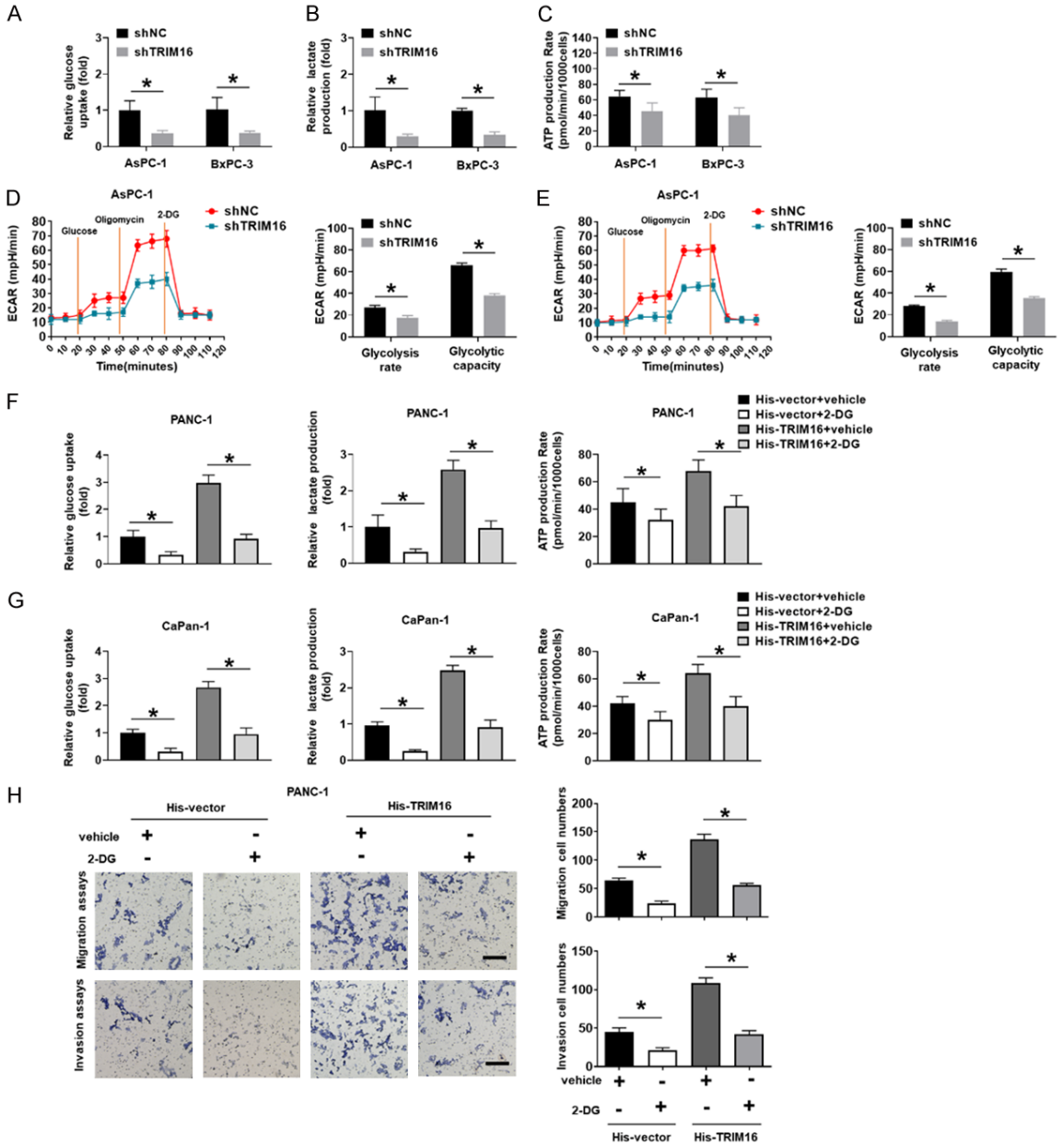
H

	Liver metastasis	*p* value
His-vector	2/6	
His-TRIM16	6/6	0.01431

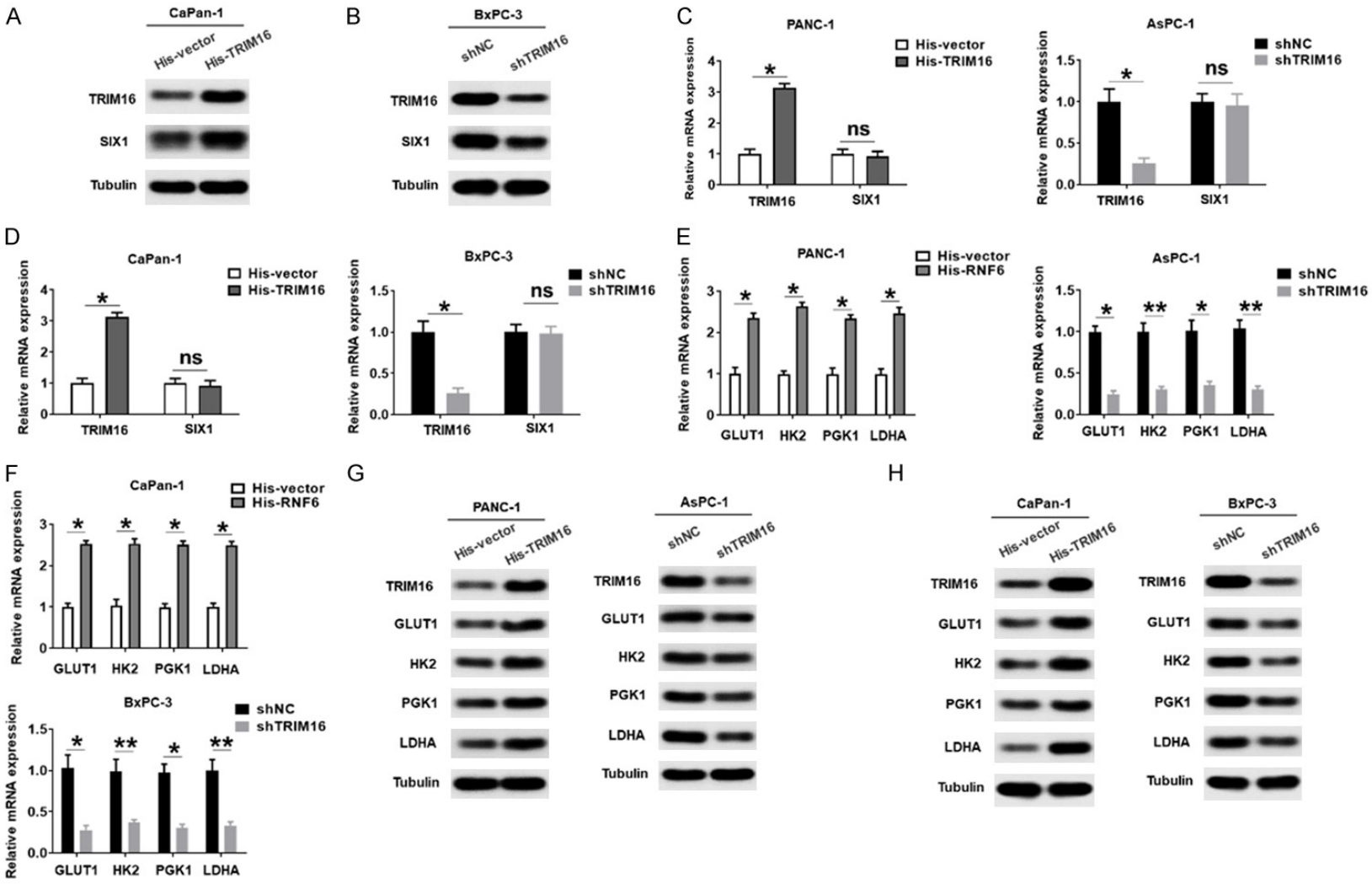
I



Supplementary Figure 3. PANC-1 and CaPan-1 cells were stably transfected with the TRIM16-overexpressing vector. A and B. Western blotting analyses were used to detect the expression level of TRIM16, Tubulin was used as a loading control. C and D. Transwell migration and transwell invasion assays were used to detect the cells' migration and invasion abilities. The image was captured at 400 × magnification. Scale bar, 50 μm. *P<0.05. E and F. RTCA assays were performed to detect the cells' metastasis ability. G. PANC-1 cells stably transfected with the TRIM16-overexpressing vector were injected into the tail vein of nude mice, and the in vivo liver metastatic signal detection were imaged by a Lumina Series III IVIS instrument. H. The incidence of liver metastasis were measured after 6-8 weeks. n = 6, **P<0.01. I. Representative image (left; magnification: × 100) and quantification (right) of H&E staining of liver metastatic nodules. n = 6. Scale bar, 50 μm. *P<0.05.



Supplementary Figure 4. A-C. Glucose consumption, lactate production, and ATP levels in AsPC-1 and BxPC-3 cells stably transfected with the TRIM16-silenced vector. Three independent experiments were performed. *P<0.05. D and E. ECAR data showing the glycolytic rate and capacity in AsPC-1 and BxPC-3 cells stably transfected with the TRIM16-silenced vector. *P<0.05. F and G. Glucose consumption, lactate production, and ATP levels were measured in TRIM16-overexpressing PANC-1 and CaPan-1 cells with or without 2-DG. H. Transwell migration and transwell invasion assays of CaPan-1 cells transfected with TRIM16-overexpressing vector with or without 2-DG. The image was captured at 400 × magnification. Scale bar, 50 μm. *P<0.05.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CaPan-1
His-vector
His-TRIM16
TRIM16
SIX1
Tubulin
B
BxPC-3
shNC
shTRIM16
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Tubulin
C
PANC-1
His-vector
His-TRIM16
Relative mRNA expression
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SIX1
AsPC-1
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D
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E
PANC-1
His-RNF6
AsPC-1
GLUT1
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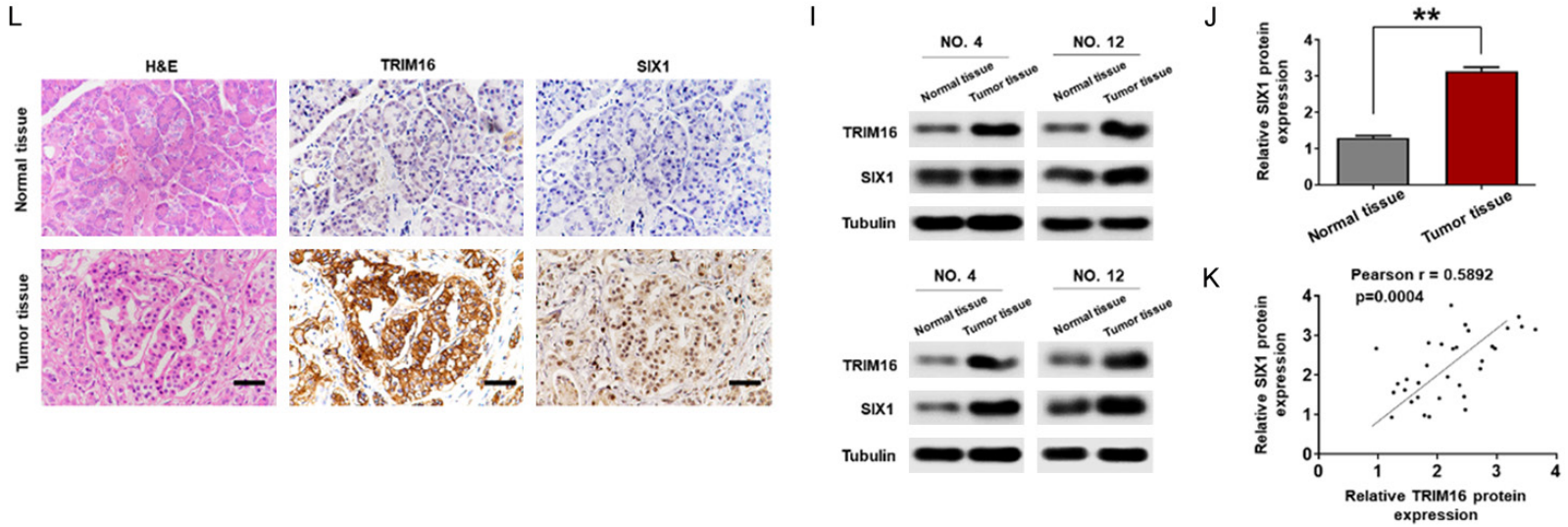
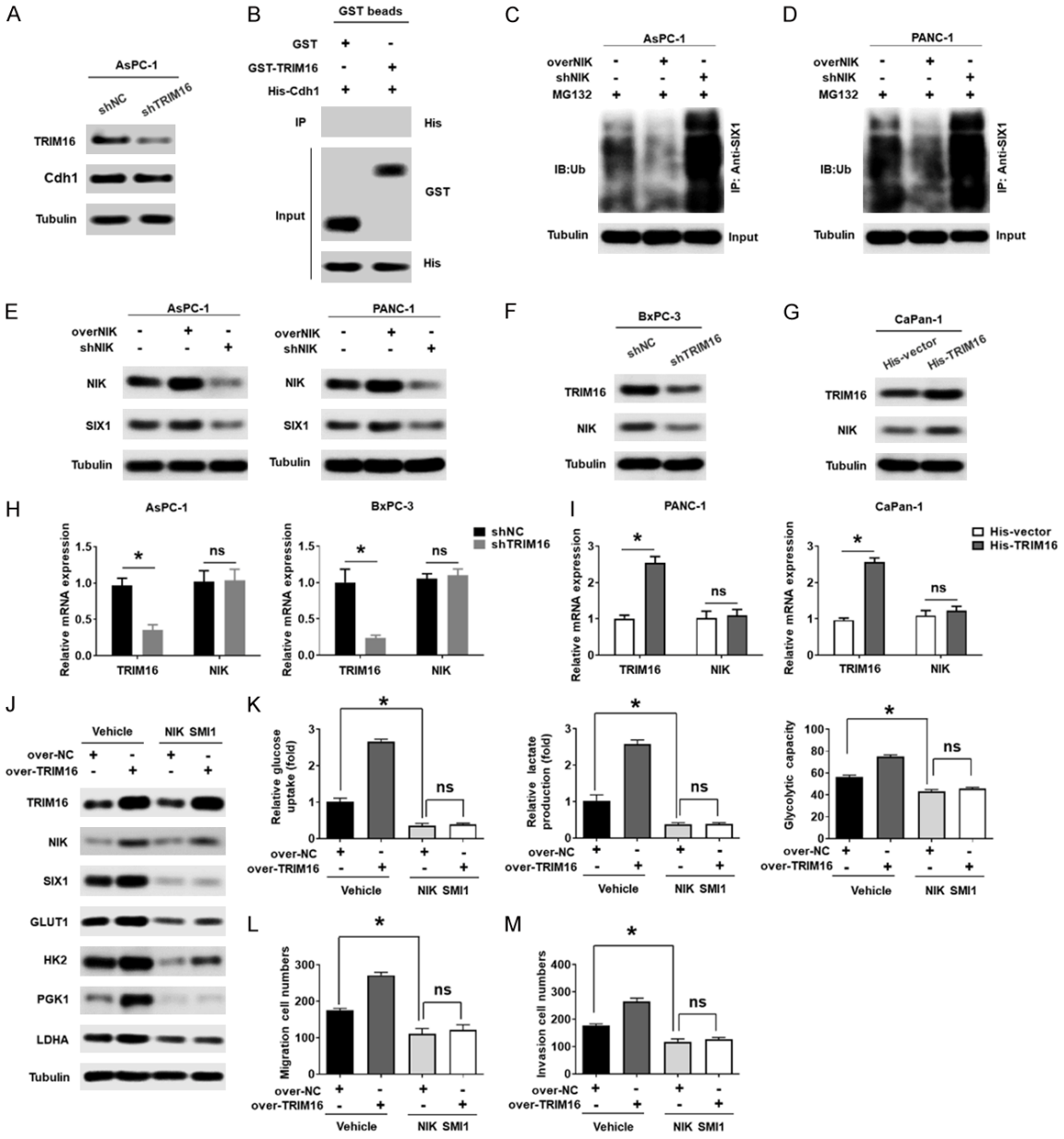
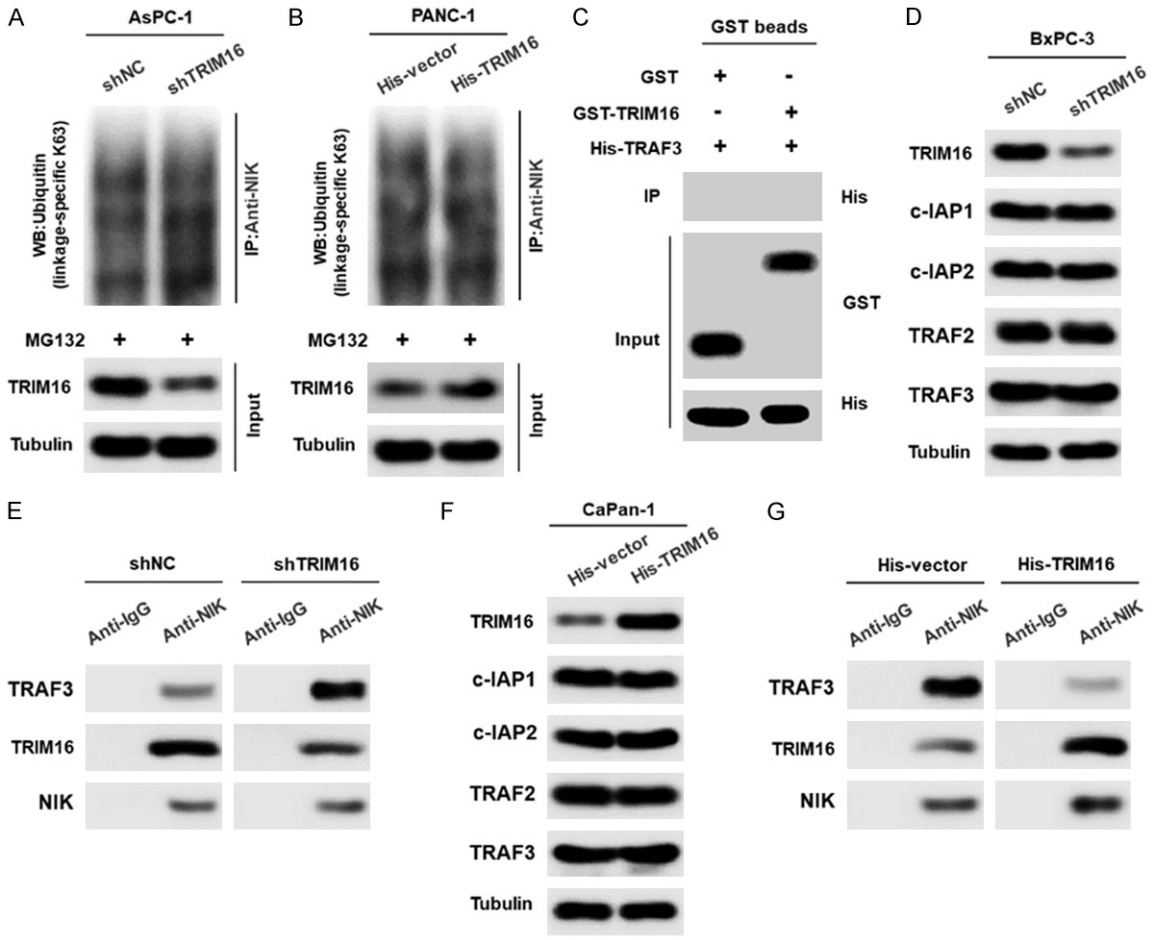


Supplementary Figure 5. A. Protein levels of SIX1 in CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. B. Protein levels of SIX1 in BxPC-3 cells transfected with TRIM16-silenced vector. C and D. MRNA levels of SIX1 in AsPC-1 or BxPC-3 cells transfected with TRIM16-silenced vector and PANC-1 or CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. *P<0.05, ns, no significant. E. MRNA levels of SIX1 target glucose metabolism genes (GLUT1, HK2, PGK1 and PFKM) in AsPC-1 cells transfected with TRIM16-silenced vector and PANC-1 cells stably transfected with the TRIM16-overexpressing vector. *P<0.05, **P<0.01. F. MRNA levels of SIX1 target glucose metabolism genes (GLUT1, HK2, PGK1 and PFKM) in BxPC-3 cells transfected with TRIM16-silenced vector and CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. *P<0.05, **P<0.01. G. Protein levels of GLUT1, HK2, PGK1 and PFKM in AsPC-1 cells transfected with TRIM16-silenced vector and PANC-1 cells stably transfected with the TRIM16-overexpressing vector. H. Protein levels of GLUT1, HK2, PGK1 and PFKM in BxPC-3 cells transfected with TRIM16-silenced vector and CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. I and J. Determination and quantification of SIX1 protein levels in pancreatic cancer tissues and paired normal tissues by western blotting assay. Tubulin was used as a loading control. K. Scatter plots show a positive correlation between TRIM16 and SIX1 at the protein level in pancreatic cancer tissues. L. Representative IHC staining of TRIM16 and SIX1 in pancreatic cancer tissues and paired normal tissues. Magnification, T 400 ×. Scale bar, 50 μm.



Supplementary Figure 6. A. Protein levels of Cdh1 in AsPC-1 cells transfected with TRIM16-silenced vector. B. GST pull-down assay performed to detect the direct interaction between TRIM16 and Cdh1. C and D. NIK inhibits ubiquitination of SIX1. AsPC-1 and PANC-1 cells were stably transfected with the NIK-silenced or NIK-overexpressing vector. The level of ubiquitin-attached SIX1 was precipitated with anti-SIX1 after the cells were treated with MG132 for 12 h, followed by western blot to detect ub. E. NIK upregulates SIX1 protein. AsPC-1 and PANC-1 cells were stably transfected with the NIK-silenced or NIK-overexpressing vector. The protein level of SIX1 was detected by using western blot. F. Protein levels of NIK in BxPC-3 cells transfected with TRIM16-silenced vector. G. Protein levels of NIK in CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. H and I. MRNA levels of NIK in AsPC-1 or BxPC-3 cells transfected with TRIM16-silenced vector and PANC-1 or CaPan-1 cells stably transfected with the TRIM16-overexpressing vector. *P<0.05, ns, no significant. J. The protein level of TRIM16, NIK, SIX1, GLUT1, HK2, PGK1 and LDHA were detected by western blot in TRIM16-overexpressing CaPan-1 cells with or without NIK SMI1. K. Glucose consumption, lactate production and glycolytic capacity were measured in TRIM16-overexpressing CaPan-1 cells with or without NIK SMI1. *P<0.05, ns, no significant. L and M. The cells' migration and invasion abilities were measured by using transwell assays in TRIM16-overexpressing CaPan-1 cells with or without NIK SMI1. *P<0.05, ns, no significant.



Supplementary Figure 7. A and B. AsPC-1 cells were stably transfected with the TRIM16-silenced vector, PANC-1 cells were stably transfected with the TRIM16-overexpressing vector. The level of ubiquitin-attached NIK was precipitated with anti-NIK after the cells were treated with MG132 for 12 h, followed by western blot to detect ub (linkage-specific K63). C. GST pull-down assay performed to detect the direct interaction between TRIM16 and TRAF3. D. The protein level of TRIM16, c-IAP1, c-IAP2, TRAF2 and TRAF3 were detected by using western blot in TRIM16-silenced BxPC-3 cells. E. The binding amount of NIK with TRAF3 or TRIM16 were detected by immunoprecipitation with anti-NIK and blots were probed as indicated antibodies in TRIM16-silenced BxPC-3 cells. F. The protein level of TRIM16, c-IAP1, c-IAP2, TRAF2 and TRAF3 were detected by using western blot in TRIM16-overexpressing CaPan-1 cells. G. The binding amount of NIK with TRAF3 or TRIM16 were detected in TRIM16-overexpressing CaPan-1 cells.