USP21 deubiquitinase promotes pancreas cancer cell stemness via Wnt pathway activation

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Supplemental Materials

Supplemental Figures S1-6

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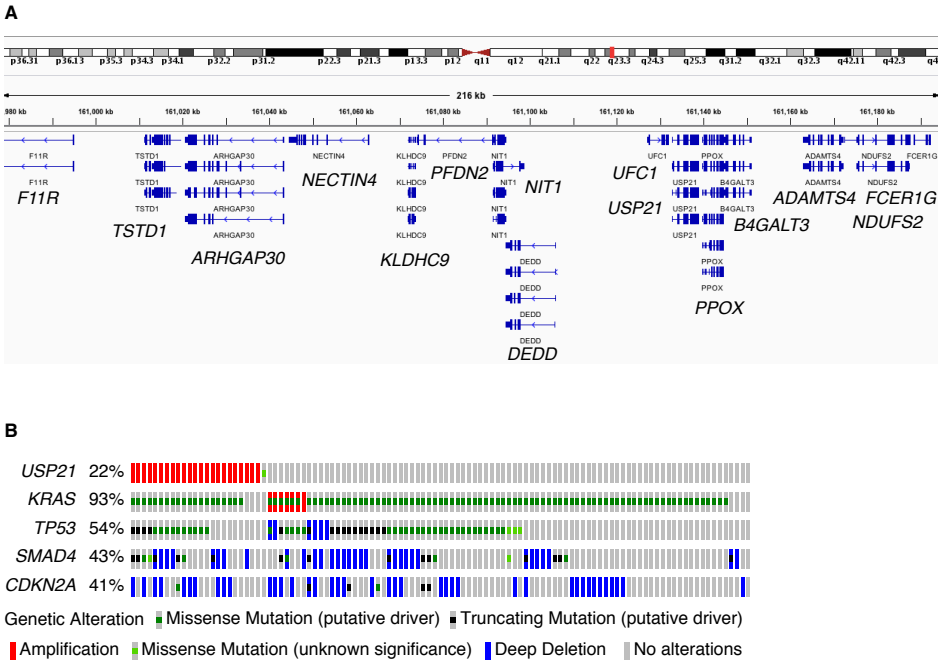
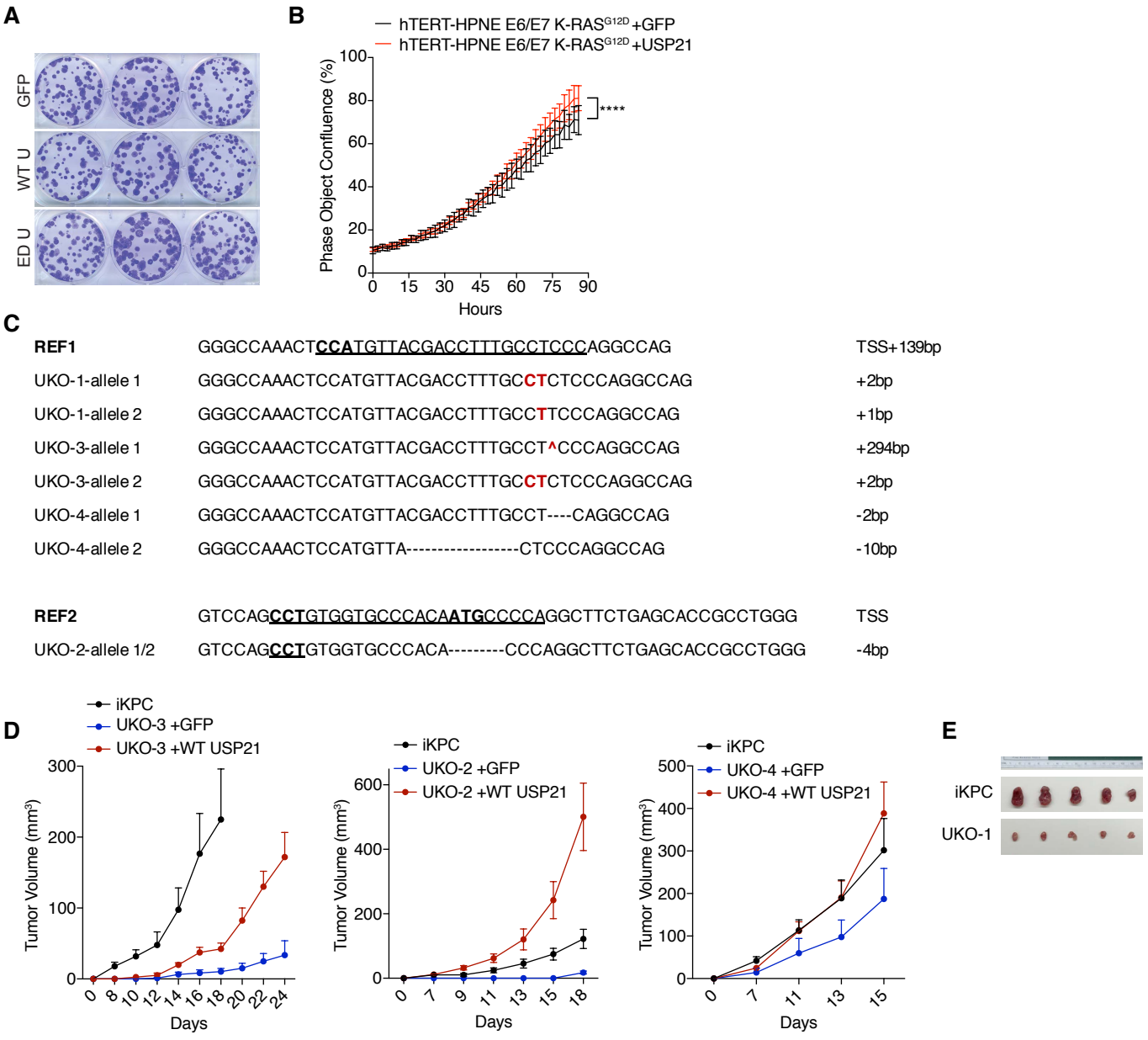
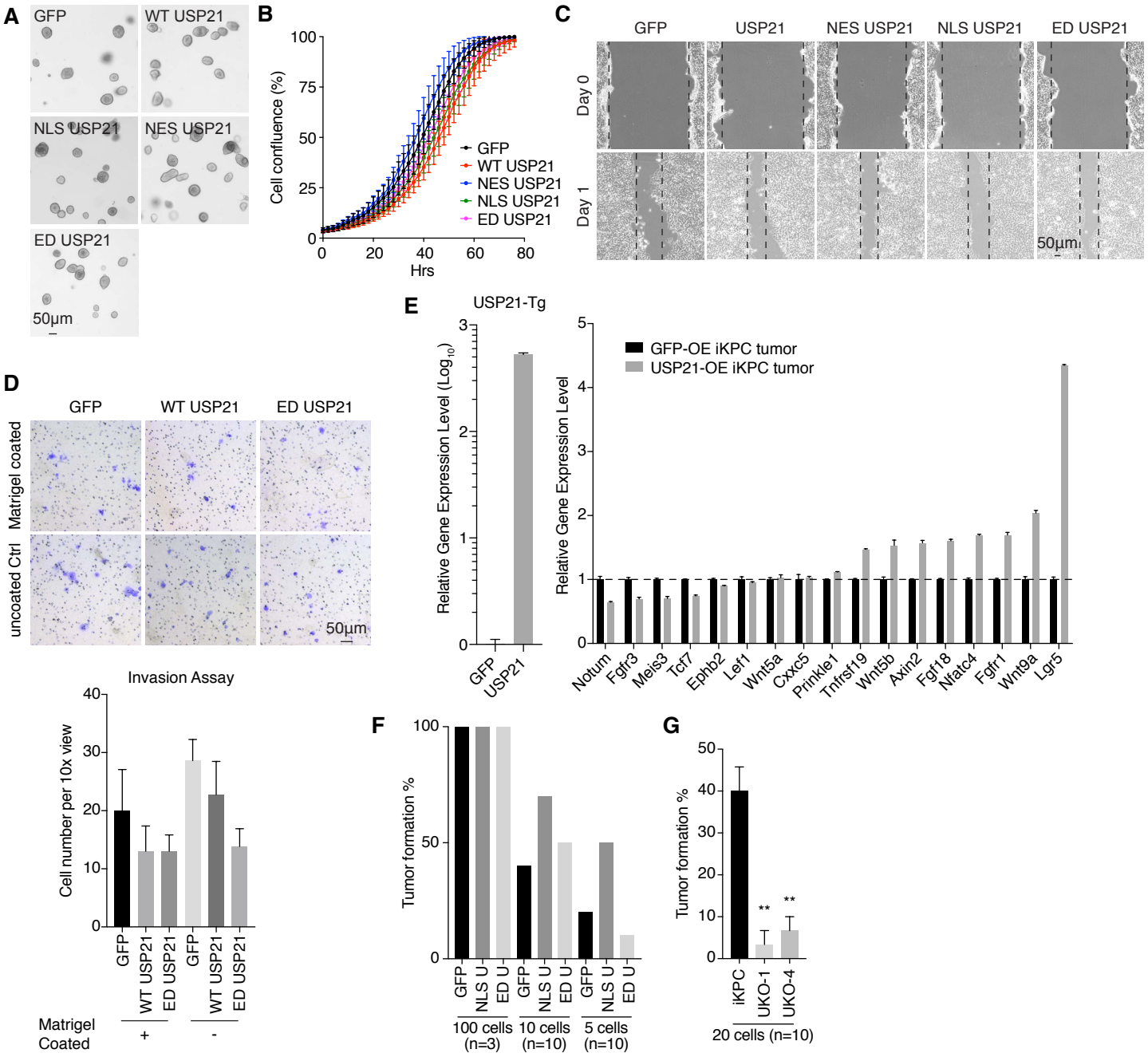


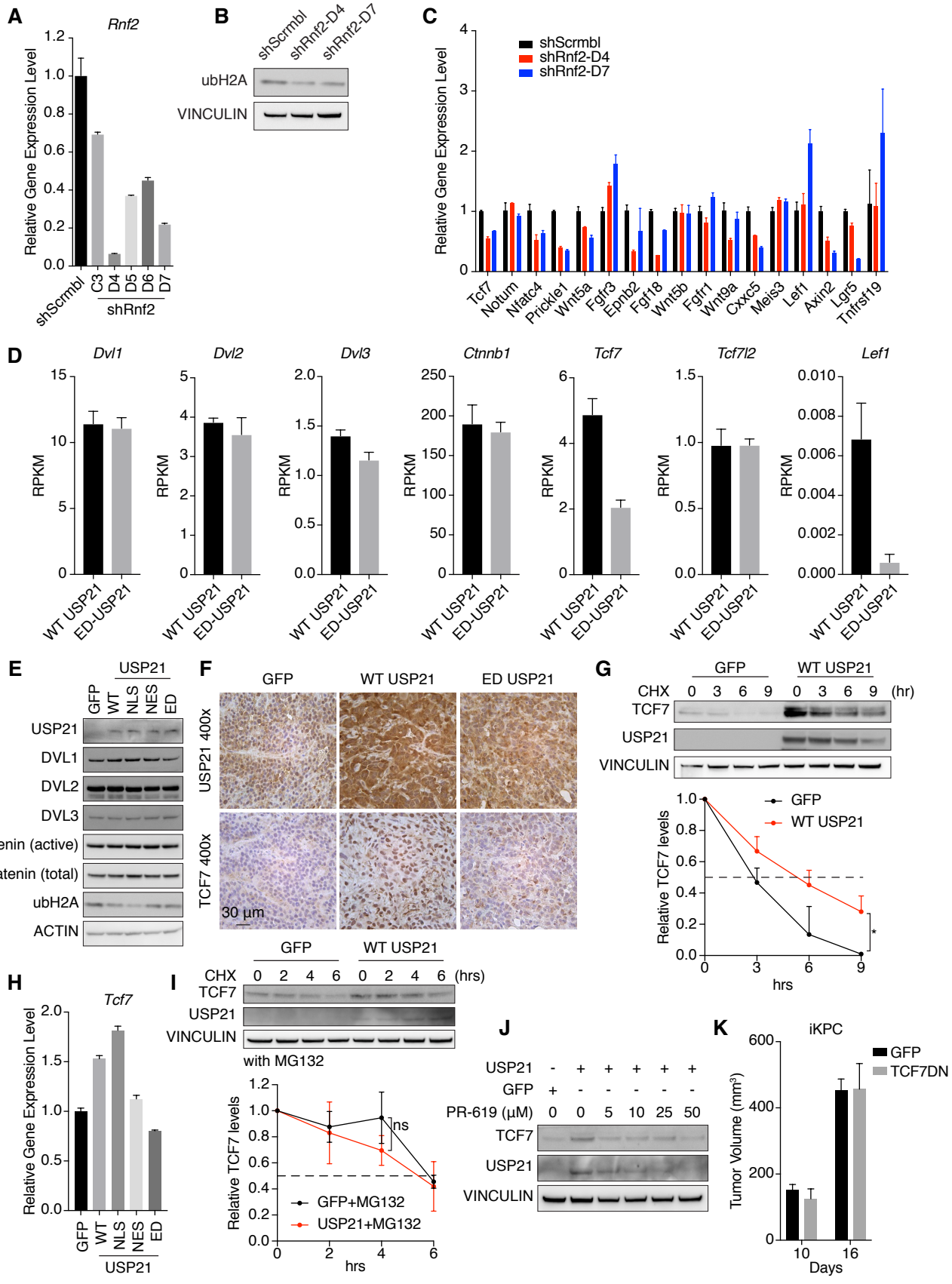
Supplemental Fig. S1. Genetic analysis of *USP21* in human PDAC datasets. (**A**) Genes located in the minimal common region of the *USP21* amplicon. (**B**) Genetic alterations of *USP21* and other common driver genetic events in PDAC (UTSW dataset).



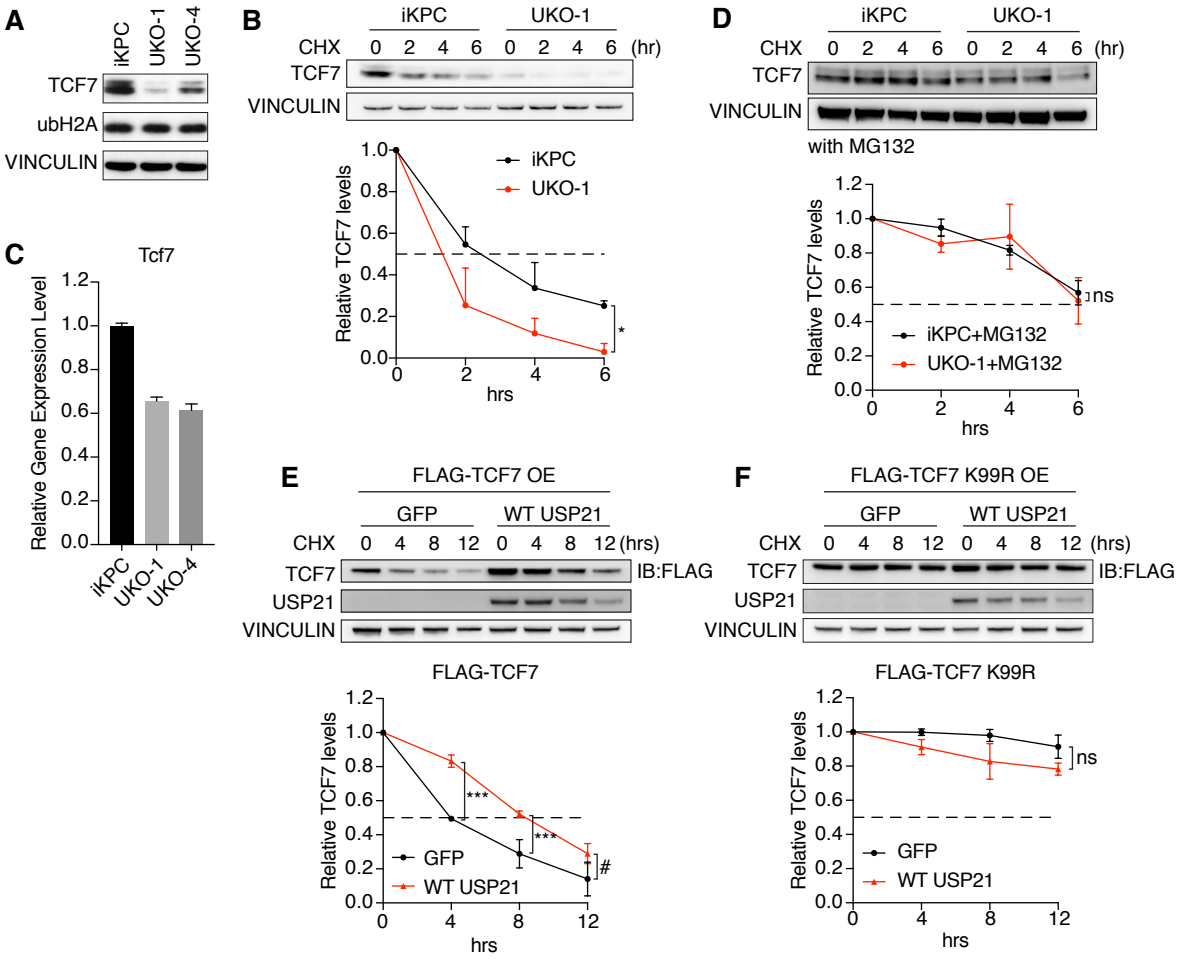
Supplemental Fig. S2. Functional analysis of USP21. (**A**) Colony formation assay comparing GFP, WT-USP21 (WT U) and ED-USP21 (ED U) OE iKPC cells. 500 cells per well were seeded in 6-well plates and stained with crystal violet after 1 week. (**B**) Cell proliferation comparing GFP and WT-USP21 OE hTERT-HPNE E6/E7-KRAS^{G12D} cells (n=6). (**C**) The sgRNA design to knockout *Usp21* in iKPC cells by CRISPR/Cas9. (**D**) Knockout of *Usp21* impaired iKPC tumor growth *in vivo* (n=5). (**E**) Comparison of the tumor sizes generated from iKPC cells and UKO-1 cells. For **B** and **D**, data are represented as mean ± SEM.



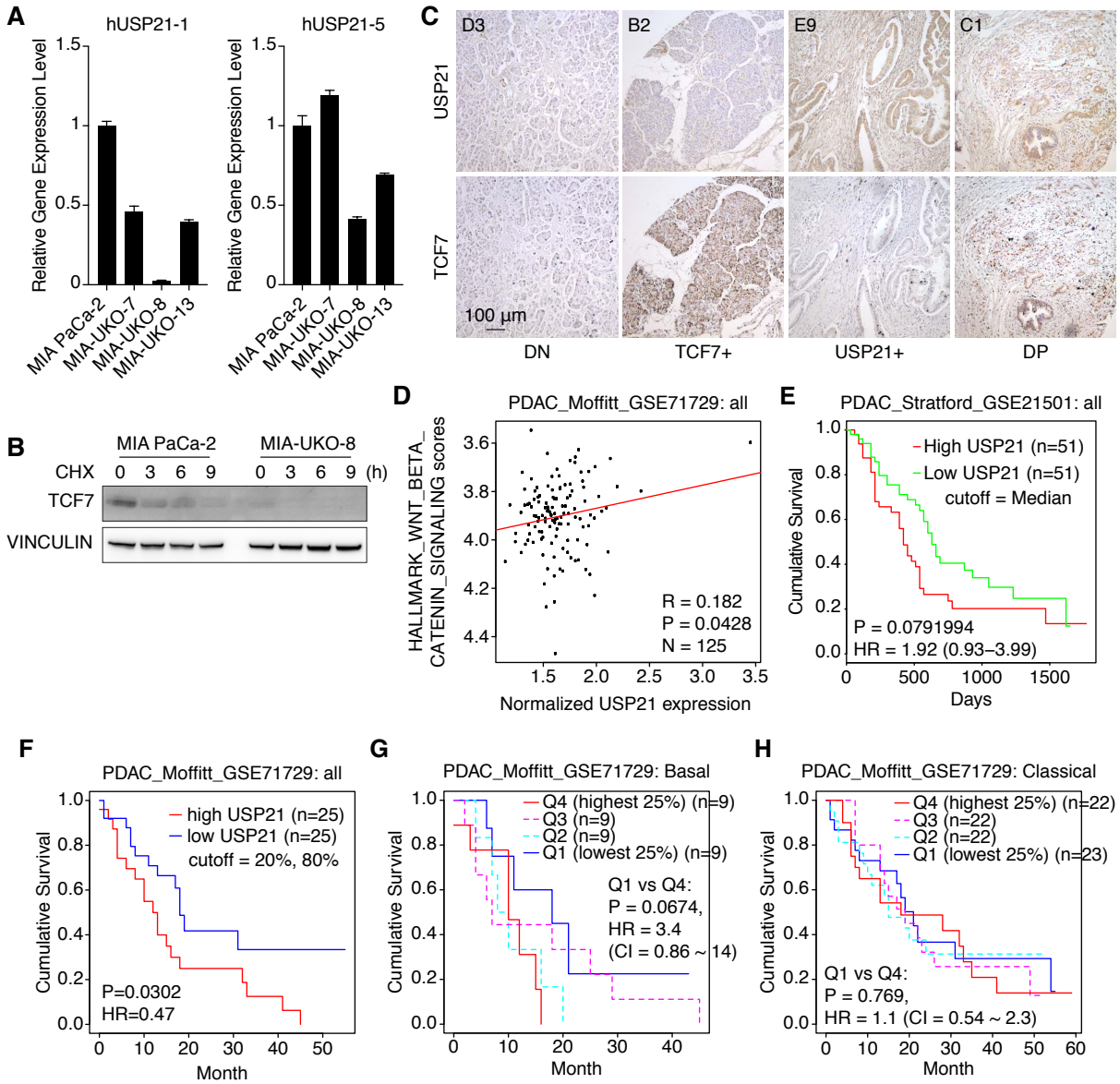
Supplemental Fig. S3. Comparison of tumorigenic functions between USP21 variants. (**A-C**) Spheroid colony formation in 3-D culture (A), cell proliferation assay (B) and cell migration assay (C) comparing iKPC cells overexpressing GFP and USP21 variants. (**D**) Cell invasion assay comparing GFP, WT- and ED-USP21 OE iKPC cells. Representative images of crystal violet staining of invaded cells and quantification were shown. (**E**) RT-PCR analysis of Wnt pathway genes comparing GFP and WT-USP21 OE iKPC tumors. (**F**) Limited dilution assay comparing GFP, NLS-USP21 (NLS U) and ED-USP21 (ED U) OE iKPC cells. 100, 10 and 5 cells were injected to compare the tumor formation rate. (**G**) Limited dilution assay comparing tumor formation capacity from 20 cells between Usp21 WT and KO iKPC cells. n=3 independent experiments. For B and G, data are represented as mean ± SEM. For D and E, data are represented as mean ± SD.


A
Rnf2
Relative Gene Expression Level
shScrmbl
C3
D4
D5
D6
D7
shRnf2
B
shScrmbl
shRnf2-D4
shRnf2-D7
ubH2A
VINCULIN
C
Relative Gene Expression Level
shScrmbl
shRnf2-D4
shRnf2-D7
Tcf7
Notum
Nfatc4
Prickle1
Wnt5a
Fgfr3
Epnb2
Fgf18
Wnt5b
Fgfr1
Wnt9a
Cxxc5
Meis3
Lef1
Axin2
Lgr5
Tnfrsf19
D
Dvl1
Dvl2
Dvl3
Ctnnb1
Tcf7
Tcf7l2
Lef1
RPKM
WT USP21
ED-USP21
E
USP21
GFP
WT
NLS
NES
ED
USP21
DVL1
DVL2
DVL3
β-catenin (active)
β-catenin (total)
ubH2A
ACTIN
F
GFP
WT USP21
ED USP21
USP21 400x
TCF7 400x
30 μm
G
GFP
WT USP21
CHX
0 3 6 9 0 3 6 9 (hr)
TCF7
USP21
VINCULIN
Relative TCF7 levels
GFP
WT USP21
hrs
*
H
Tcf7
Relative Gene Expression Level
GFP
WT
NLS
NES
ED
USP21
I
GFP
WT USP21
CHX
0 2 4 6 0 2 4 6 (hrs)
TCF7
USP21
VINCULIN
with MG132
Relative TCF7 levels
ns
GFP+MG132
USP21+MG132
hrs
J
USP21
GFP
PR-619 (μM)
0 0 5 10 25 50
TCF7
USP21
VINCULIN
K
iKPC
GFP
TCF7DN
Tumor Volume (mm³)
Days

Supplemental Fig. S4. USP21 activates Wnt pathway genes via TCF7 and independent of ubH2A deubiquitination. (**A**) Validation of *Rnf2* knockdown by shRNA (n=3). (**B**) Western blot analysis of ubH2A after *Rnf2* knockdown in iKPC cells. (**C**) RT-PCR analysis of Wnt pathway genes comparing scramble control and *Rnf2* knockdown in iKPC cells (n=3). (**D**) Reads Per Kilobase of transcript, per Million mapped reads (RPKM) of *Dvl1*, *Dvl2*, *Dvl3*, *Ctnnb1*, *Tcf7*, *Tcf7l2* and *Lef1* extracted from RNA-seq data comparing WT- and ED-USP21 OE iKPC cells (n=4). (**E**) Western blot analysis of Wnt pathway proteins and ubH2A comparing iKPC cells overexpressing GFP and USP21 variants. (**F**) TCF7 protein levels in GFP, WT- and ED-USP21 OE iKPC tumors by IHC. (**G**) CHX assay of TCF7 in GFP and WT-USP21 OE iKPC cells (n=3). (**H**) *Tcf7* mRNA levels in iKPC cells overexpressing GFP and USP21 variants (n=3). (**I**) CHX assay of TCF7 in GFP and WT-USP21 OE iKPC cells with MG132 treatment (n=3). (**J**) Comparison of TCF7 protein levels in USP21 OE iKPC cells under DUB inhibitor PR-619 treatment at different concentrations. (**K**) Tumor growth comparison of GFP and TCF7DN OE iKPC cells (n=5). For **A-B**, and **G-I**, data are represented as mean ± SD. For **D** and **K**, data are represented as mean ± SEM.



Supplemental Fig. S5. Depletion of *Usp21* in iKPC cells destabilizes TCF7 and impairs tumor initiating capacity. (**A**) TCF7 and ubH2A protein levels in *Usp21* WT and KO iKPC cells by western blot analysis. (**B**) CHX assay of TCF7 in *Usp21* WT and KO iKPC cells (n=2). (**C**) *Tcf7* mRNA levels in *Usp21* WT and KO iKPC cells by RT-PCR analysis. (**D**) CHX assay of TCF7 in *Usp21* WT and KO iKPC cells with MG132 treatment (n=3). (**E**) CHX assay of exogenous FLAG-tagged TCF7 in GFP and USP21 OE 293T cells (n=3). (**F**) CHX assay of exogenous K99R mutated TCF7 in GFP and USP21 OE 293T cells (n=2). For **B-F**, data are represented as mean ± SD.



Supplemental Fig. S6. Correlation between USP21 and TCF7 in human PDAC samples. (**A**) Validation of *USP21* knockout in 3 MIA PaCa-2 single cell colonies by RT-PCR using 2 different primers. (**B**) CHX assay of TCF7 in *USP21* WT and KO Mia PaCa-2 cells. (**C**) Representative images of DN, TCF7+, USP21+ and DP human TMA samples (from left to right: inflammation, normal pancreas, PDAC, PDAC, respectively). (**D**) Correlation analysis of Hallmark of Wnt pathway gene signature and *USP21* expression in Moffitt PDAC dataset. (**E**, **F**) Kaplan-Meier survival analysis based on *USP21* expression in Stratford PDAC dataset (E) and Moffitt PDAC dataset (F). (**G**, **H**) Kaplan-Meier survival analysis based on *USP21* expression in basal-like subtype (G) and classical subtype (H) in Moffitt PDAC dataset.

Supplementary Table 1. DNA sequence information of protein tags, sgRNA, primers and shRNA

ID	Gene ID	Sequence	Note
MAPKK nuclear export signal		CTTCAAAAAAAACTTGAAGAACTTGAACTT	
SV40 nuclear localization signal		AGCCCAAAGAAGAAGAGAAAAGTAGAA	
HA tag		TACCCATACGATGTTCCAGATTACGCT	
FLAG tag		GACTACAAAGACGATGACGACAAG	
USP21 sgRNA target	NM_001014443	GTTAATATCCAGCCCCGAGT	sgRNA
hUSP21-1F	NM_001014443	TAATATCCAGCCCCGAGTGG	shRNA validation
hUSP21-1R	NM_001014443	TAACATGGGGTTTGGCCCAG	shRNA validation
hUSP21-5F	NM_001014443	AGCCACCTGTTAATATCCAGCC	shRNA validation
hUSP21-5R	NM_001014443	CAGAGGTCGTAACATGGGGT	shRNA validation
TCF7-K52R-F	XM_006532800	CCTGGCAGAGCTCAGGTCCTCACTGGTGA	TCF7 site mutation
TCF7-K52R-R	XM_006532800	TCACCAGTGAGGACCTGAGCTCTGCCAGG	TCF7 site mutation
TCF7-K99R-F	XM_006532800	GAGACTTTTCCCGGACAGACTTCCAGAGTCCC	TCF7 site mutation
TCF7-K99R-R	XM_006532800	GGGACTCTGGAAGTCTGTCCGGGAAAAGTCTC	TCF7 site mutation
TCF7-K277R-F	XM_006532800	GCCCTCCTCAGGGAGGCAGGAGCTG	TCF7 site mutation
TCF7-K277R-R	XM_006532800	CAGCTCCTGCCTCCCTGAGGAGGGC	TCF7 site mutation
TCF7-K406R-F	XM_006532800	CGGTACTTACCCGGAGAGGGCCGCTGC	TCF7 site mutation
TCF7-K406R-R	XM_006532800	GCAGCGGCCCTCTCCGGGTAAGTACCG	TCF7 site mutation
shRnf2-D4	NM_011277	CCGGTGAGAAGCAGTACACCATTTACTCGAGTAAATGGTGTACTGCTTCTCATTTTTG	shRNA
shRnf2-D7	NM_011277	GTACCGGGTAATCTGTGCCATAAGATTTCTCGAGAAATCTTATGGCACAGATTACTTTTTTG	shRNA

Supplementary Table 2. Antibody information

Antibody	Application	Vendor	Cat. #
USP21	IHC	Sigma	HPA028397
USP21	WB	Santa Cruz	sc-79305
CYCLOPHILIN A	WB	CST	2175s
LAMIN B	WB	CST	13823
TCF7	WB, IHC	CST	2203S
TCF7	WB	CST	2206S
TCF7L2	WB	CST	2269S
ACTIN	WB	Sigma	A2228
FLAG	IP, WB	Sigma	F1804
HA	IP, WB	BioLegend	901501
HA	WB	CST	3724s
β-CATENIN (active)	WB	CST	8814s
β-CATENIN (total)	WB	CST	8480
DVL1	WB	Santa Cruz	sc-8025
DVL2	WB	CST	3224s
DVL3	WB	Santa Cruz	sc-8027
ubH2A	WB	CST	8240s
VINCULIN	WB	Sigma	V4505
GFP	IHC	abcam	ab6673
Ki67	IHC	Thermo Scientific	RM-9106-S1
Anti-rabbit IgG, HRP-linked	WB	CST	7074s

Anti-mouse IgG, HRP-linked	WB	CST	7076s
Rabbit anti-Goat IgG (H+L)	WB	Thermo	61-1620