Supplemental Information

for

Single copies of mutant *KRAS* and mutant *PIK3CA* cooperate in immortalized human epithelial cells to induce tumor formation

Grace M. Wang[1*], Hong-Yuen Wong[1*], Hiroyuki Konishi[1,2], Brian G. Blair[1], Abde Abukhdeir[1], John P. Gustin[1,3], D. Marc Rosen[1], Samuel Denmeade[1], Zeshaan Rasheed[1], William Matsui[1], Joseph P. Garay[1], Morassa Mohseni[1], Michaela J. Higgins[1,4], Justin Cidado[1], Danijela Jelovac[1], Sarah Croessmann[1], Rory Cochran[1], Sivasundaram Karnan[2], Yuko Konishi[2], Akinobu Ota[2], Yoshitaka Hosokawa[2], Pedram Argani[1], Josh Lauring[1], Ben Ho Park[1,3]

[1] *The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, MD*

[2] *Department of Biochemistry, Aichi Medical University School of Medicine*

Nagakute, Aichi 480-1195, Japan

[3] *The Whiting School of Engineering, Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD*

[4]*Current address: Massachusetts General Hospital Cancer Center, Boston, MA*

*Contributed equally to this work

Address correspondence to:

Ben Ho Park MD, PhD

Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University,

1650 Orleans Street, Room 151, Baltimore, MD, 21287, USA

Tel: 410-502-7399; FAX: 410-614-4073

email: bpark2@jhmi.edu

Supplemental Figures and Legends

Figure S1

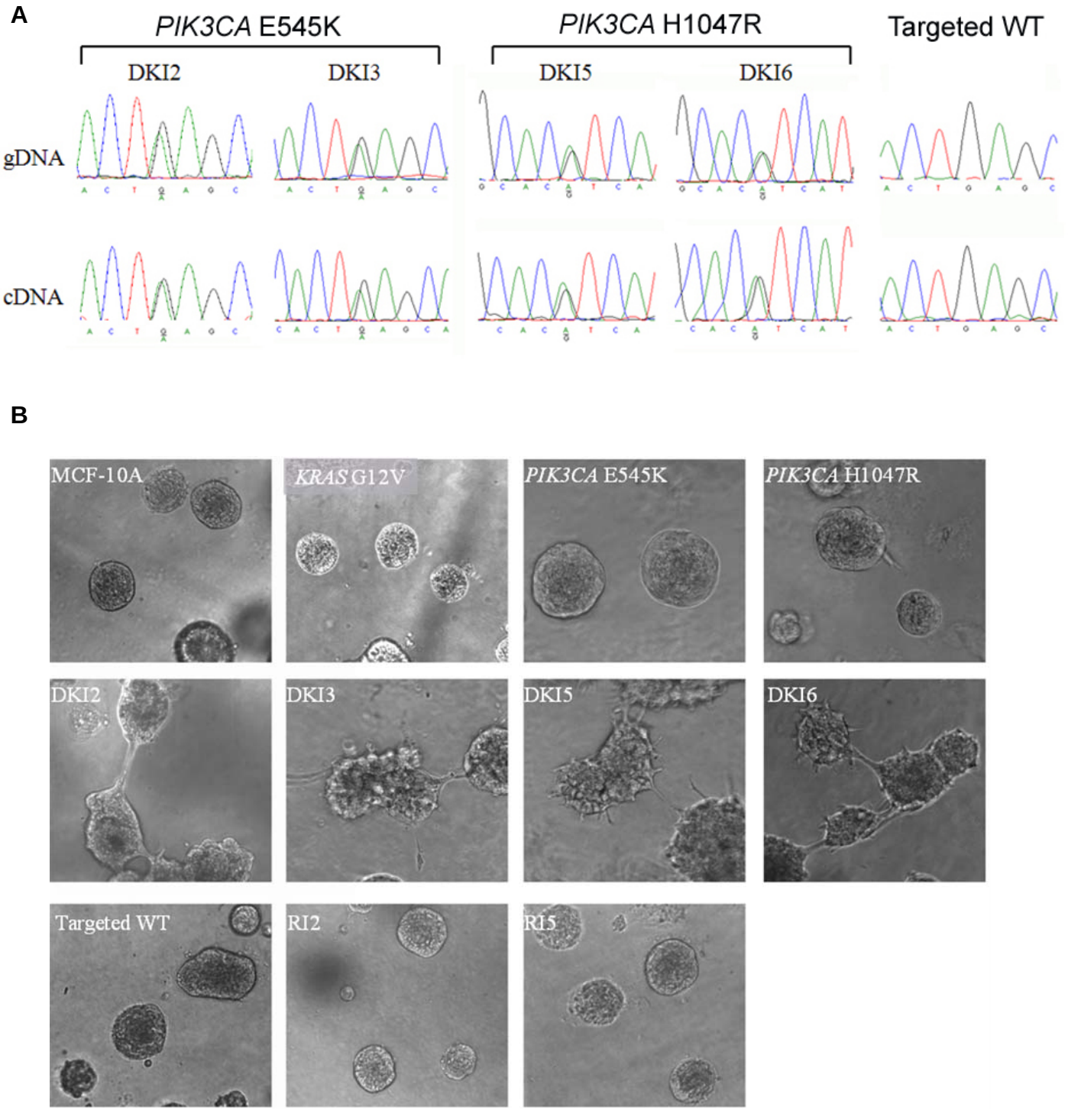


Figure S2

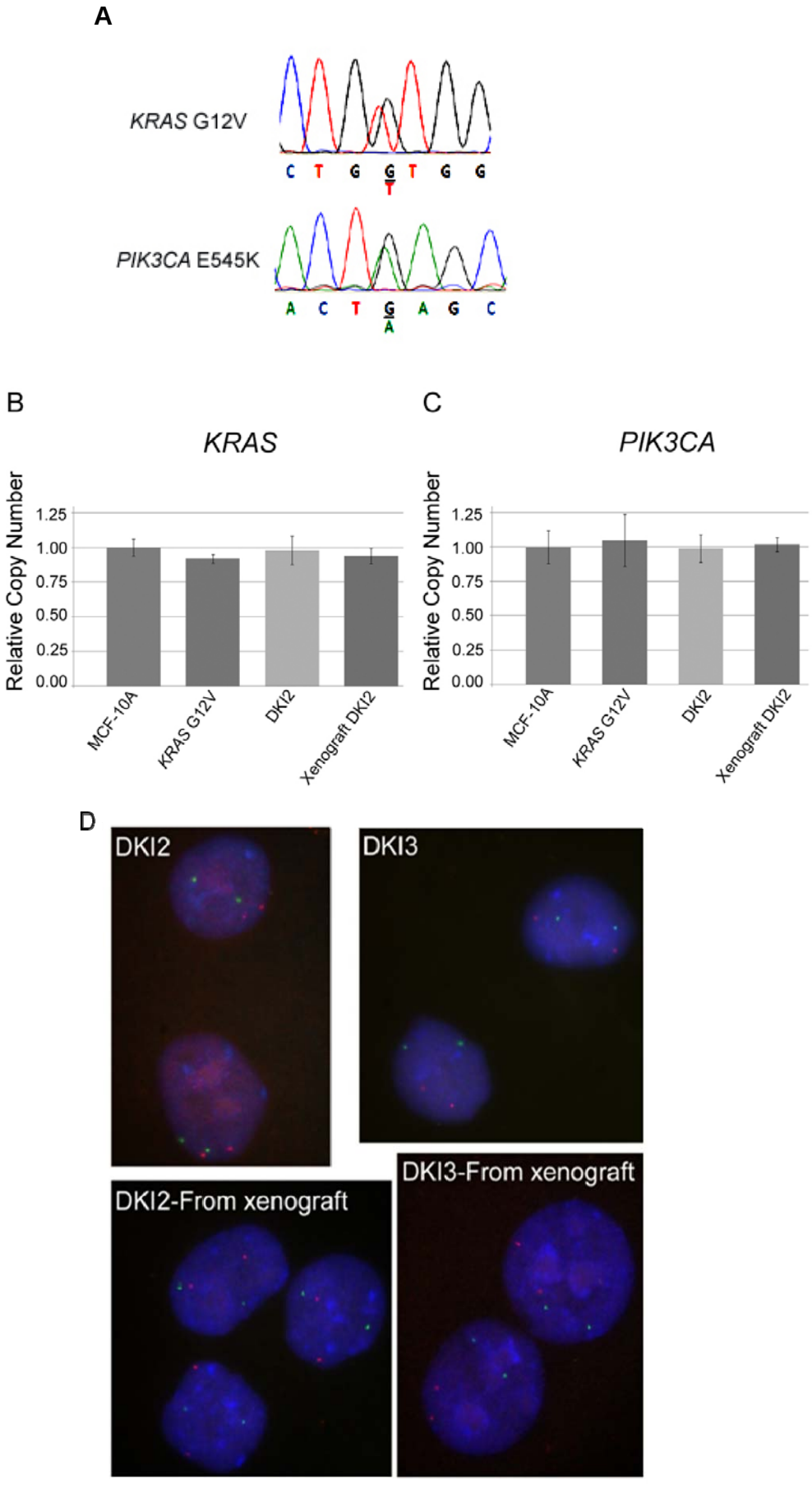

A
KRAS G12V
C T G G T G G
T
PIK3CA E545K
A C T G A G C
A
B
KRAS
Relative Copy Number
1.25
1.00
0.75
0.50
0.25
0.00
MCF-10A
KRAS G12V
DKI2
Xenograft DKI2
C
PIK3CA
Relative Copy Number
1.25
1.00
0.75
0.50
0.25
0.00
MCF-10A
KRAS G12V
DKI2
Xenograft DKI2
D
DKI2
DKI3
DKI2-From xenograft
DKI3-From xenograft

Figure S3

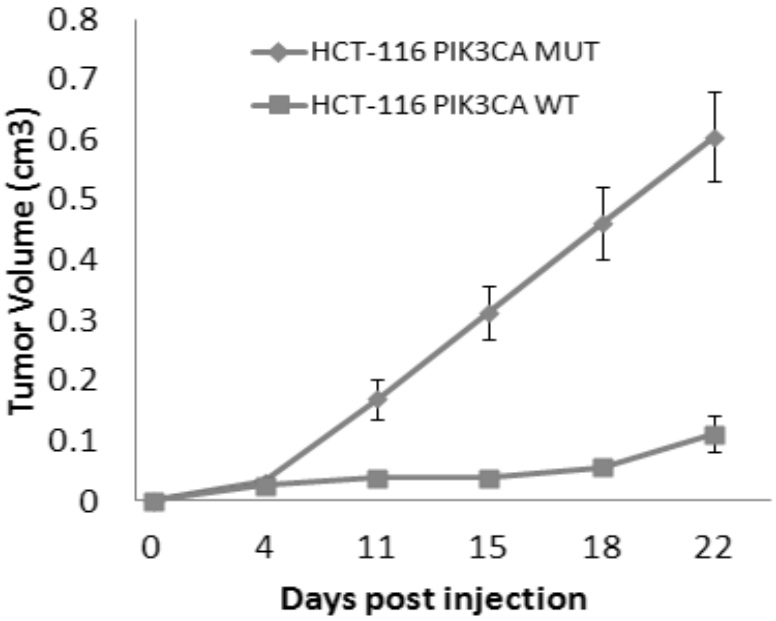

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Tumor Volume (cm3)
HCT-116 PIK3CA MUT
HCT-116 PIK3CA WT
0
4
11
15
18
22
Days post injection

Figure S4

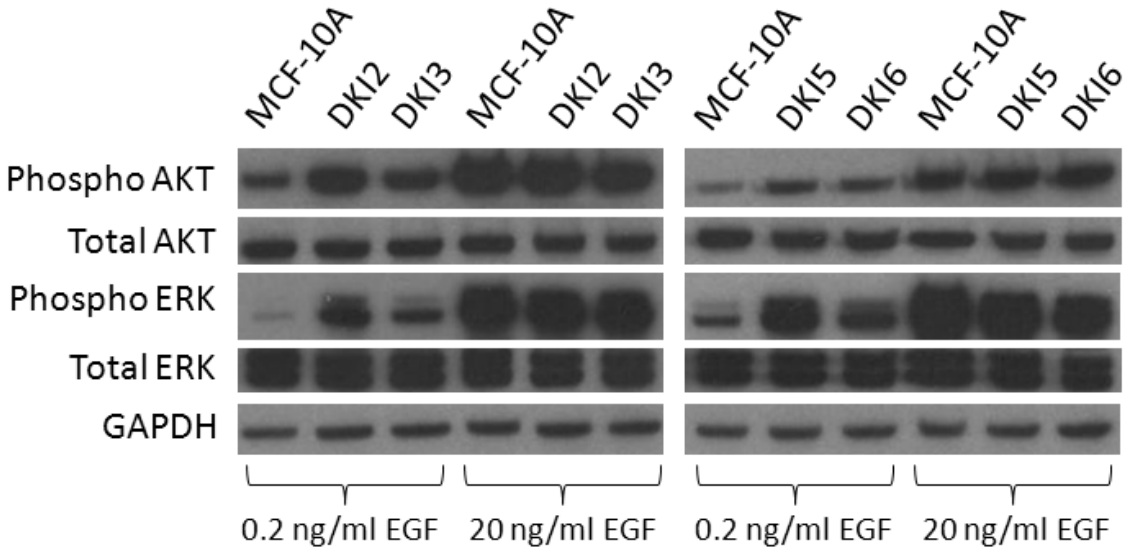

MCF-10A
DKI2
DKI3
MCF-10A
DKI2
DKI3
MCF-10A
DKI5
DKI6
MCF-10A
DKI5
DKI6
Phospho AKT
Total AKT
Phospho ERK
Total ERK
GAPDH
0.2 ng/ml EGF
20 ng/ml EGF
0.2 ng/ml EGF
20 ng/ml EGF

Figure S5

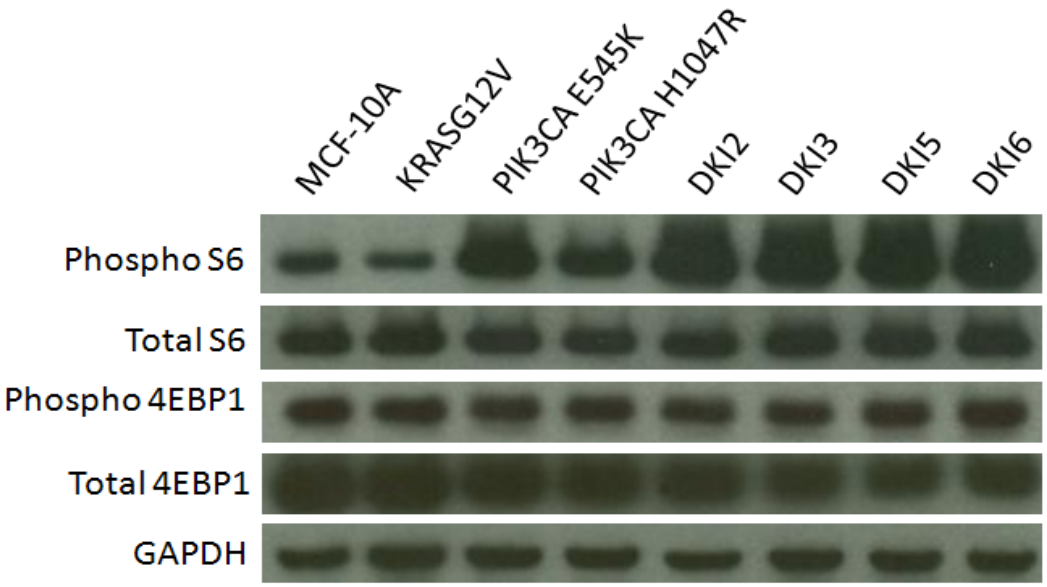

MCF-10A
KRASG12V
PIK3CA E545K
PIK3CA H1047R
DKI2
DKI3
DKI5
DKI6
Phospho S6
Total S6
Phospho 4EBP1
Total 4EBP1
GAPDH

Figure S6

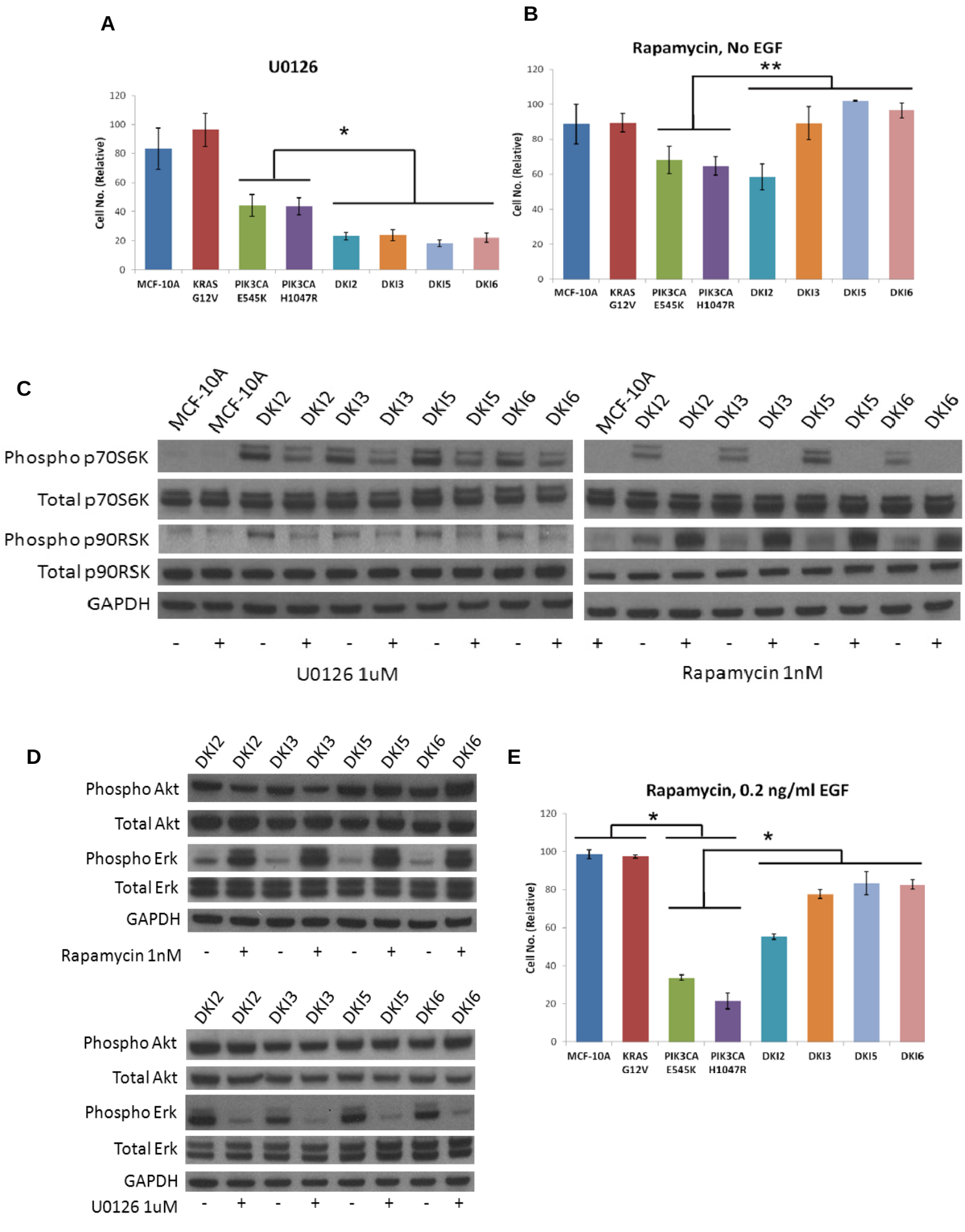
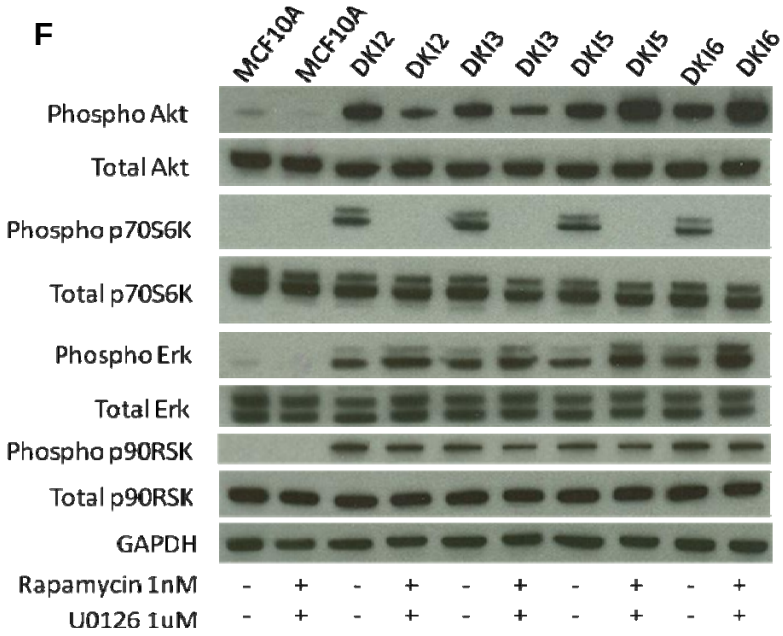
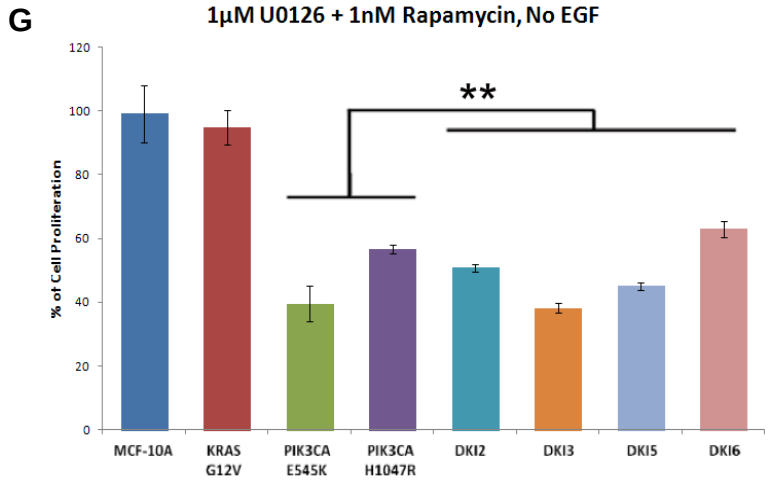

A
U0126
Cell No. (Relative)
MCF-10A
KRAS G12V
PIK3CA E545K
PIK3CA H1047R
DKI2
DKI3
DKI5
DKI6
B
Rapamycin, No EGF
C
Phospho p70S6K
Total p70S6K
Phospho p90RSK
Total p90RSK
GAPDH
U0126 1uM
Rapamycin 1nM
D
Phospho Akt
Total Akt
Phospho Erk
Total Erk
GAPDH
Rapamycin 1nM
U0126 1uM
E
Rapamycin, 0.2 ng/ml EGF

Figure S6 (cont.)





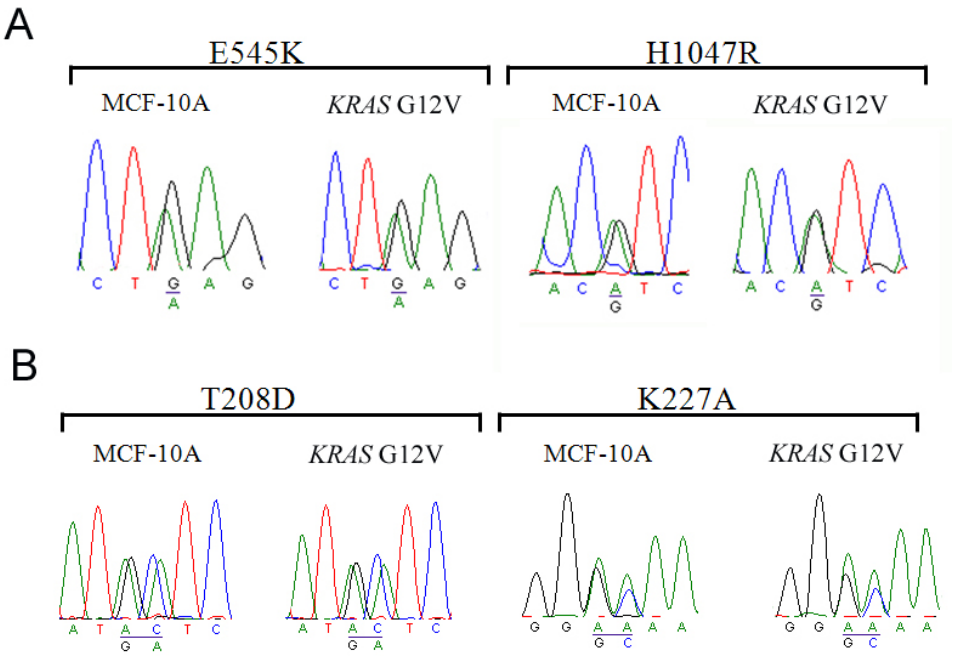
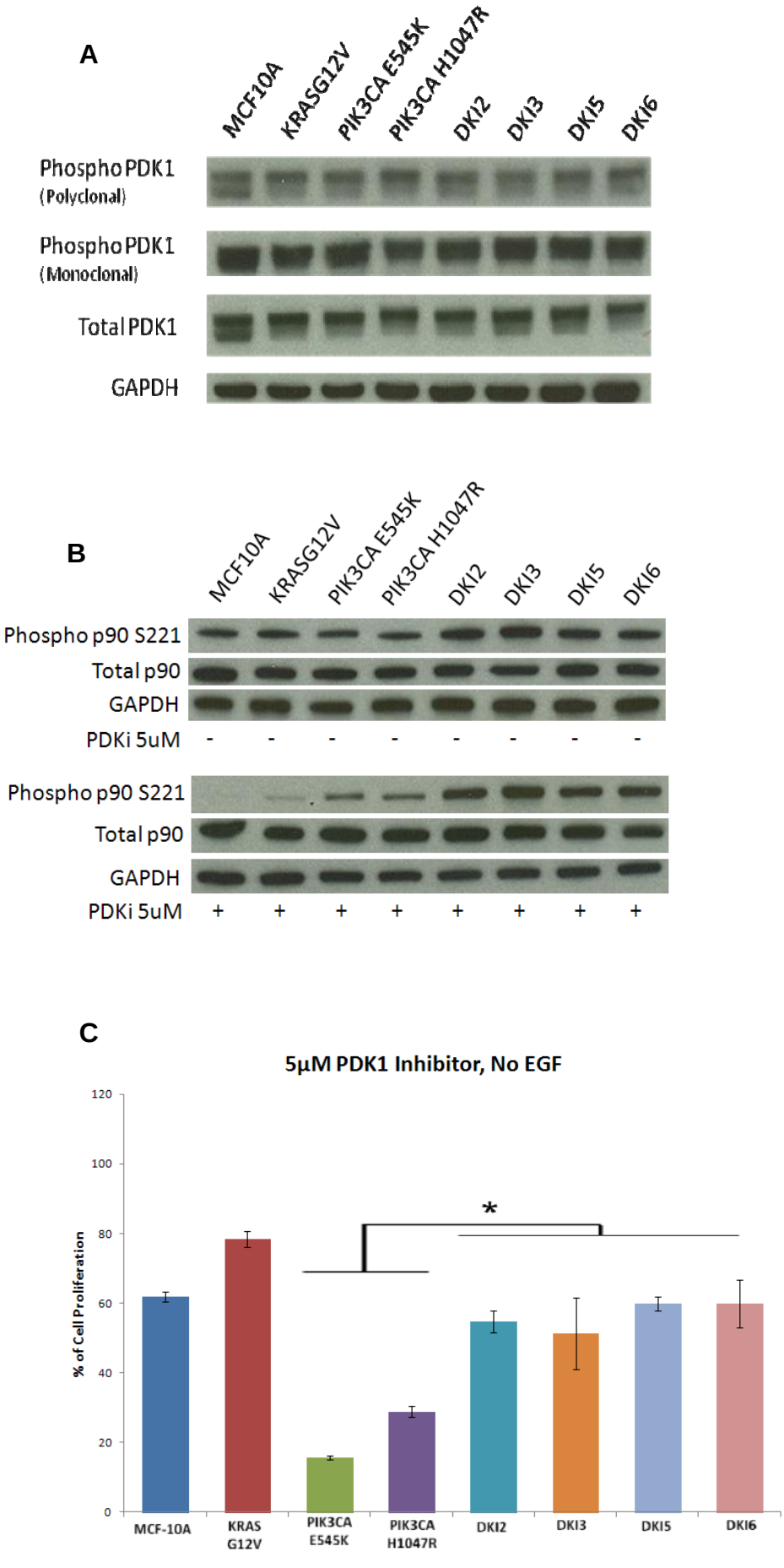
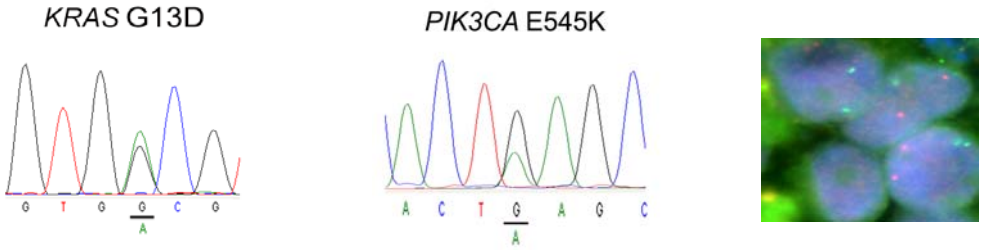

A
E545K
H1047R
MCF-10A
KRAS G12V
MCF-10A
KRAS G12V
B
T208D
K227A
MCF-10A
KRAS G12V
MCF-10A
KRAS G12V

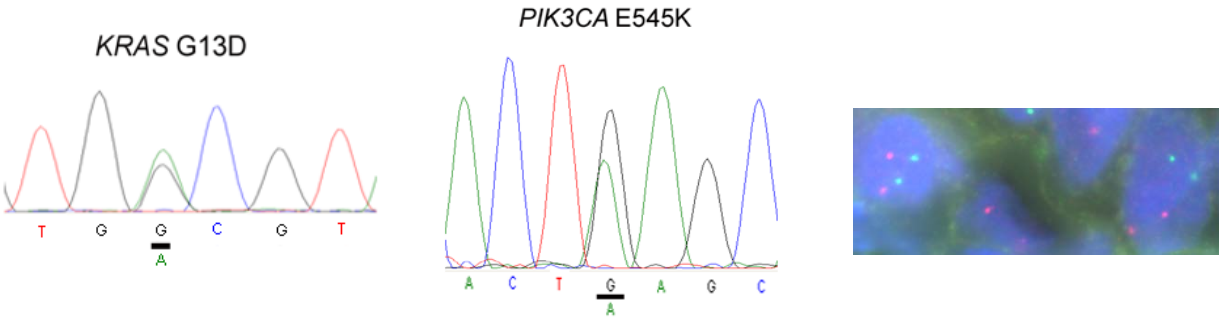
Figure S8


A
MCF10A
KRASG12V
PIK3CA E545K
PIK3CA H1047R
DKI2
DKI3
DKI5
DKI6
Phospho PDK1 (Polyclonal)
Phospho PDK1 (Monoclonal)
Total PDK1
GAPDH
B
MCF10A
KRASG12V
PIK3CA E545K
PIK3CA H1047R
DKI2
DKI3
DKI5
DKI6
Phospho p90 S221
Total p90
GAPDH
PDKi 5uM
- - - - - - - -
Phospho p90 S221
Total p90
GAPDH
PDKi 5uM
+ + + + + + + +
C
5μM PDK1 Inhibitor, No EGF
% of Cell Proliferation
120
100
80
60
40
20
0
*
MCF-10A
KRAS G12V
PIK3CA E545K
PIK3CA H1047R
DKI2
DKI3
DKI5
DKI6

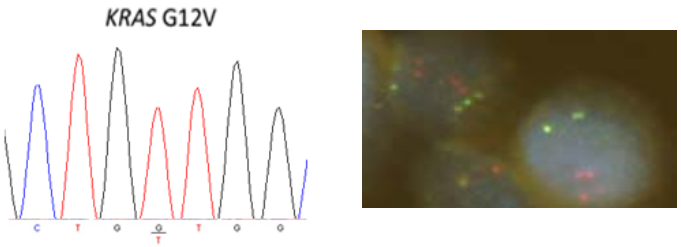
Patient Sample #3 MIN tumor



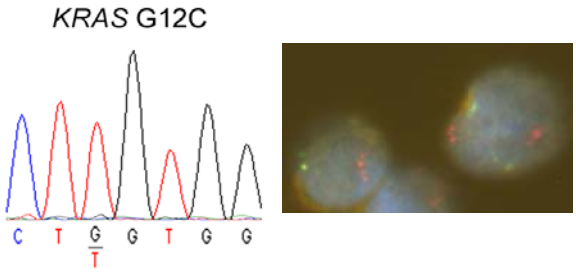
Patient Sample #4 MIN tumor



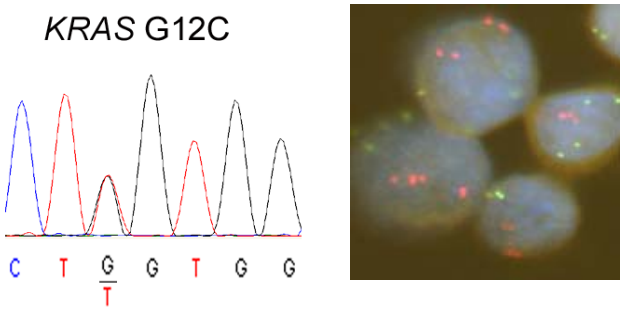
Patient Sample #1 non-MIN tumor



Patient Sample #2 non-MIN tumor



Patient Sample #4 non-MIN tumor



Supplementary Figure Legends

Figure S1. Double knock in clones have single mutant *PIK3CA* alleles and equivalent expression of mutant and wild type *PIK3CA* and form aberrant structures in 3D culture.
A, Successfully targeted knock in of mutant *PIK3CA* E545K and H1047R alleles into *KRAS* G12V cells is demonstrated by the presence of equivalent heterozygous mutant and wild type alleles in genomic DNA (gDNA) and for mRNA expression by direct sequencing of RT-PCR products (cDNA). A control clone for exon 9 gene targeting is also shown (Targeted WT) whereby gene targeting using the E545K vector was identified, but due to the cross over junction, the E545K mutation was not introduced and only wild type alleles are present. Point mutations are denoted beneath the wild-type nucleotides.
B, DKI and control cells were seeded at equal density in chamber slides within Matrigel and cultured for 21 days. Acini were visualized by contrast-phase light microscopy and photographed. Cells with both *KRAS* and *PIK3CA* knock in mutations exhibit increased acinar size and morphological changes including protrusions and bridging with neighboring acini (Bar = 100 μM).

Figure S2. DKI xenografts retain the same number of *KRAS* and *PIK3CA* alleles.
A, DKI xenografts were adapted back to culture and gDNA was extracted and used for PCR and direct sequencing. Representative sample traces from DKI2 xenograft tumor gDNA is shown for mutant *KRAS* G12V and *PIK3CA* E545K knock in alleles relative to wild type alleles. Point mutations are denoted beneath the wild-type nucleotides.
B, C, Cell line gDNA was extracted and used for quantitative real time PCR using primers listed in Table S6. Results shown are the difference in cycle numbers for *KRAS* and *PIK3CA* relative to GAPDH and then normalized to parental MCF-10A. Error bars represent SEM of triplicate samples. Representative results are shown for parental MCF-10A, *KRAS* G12V, DKI2cells and cells isolated from a DKI2 xenograft. Note: a relative change in copy number of two or greater would be indicative of a gain or loss of a *KRAS* or *PIK3CA* allele.
D, FISH showing copy numbers of *KRAS* and *PIK3CA.* Representative cells are shown for DKI clones before (top panels) and after (bottom) inoculation and recovery of xenografts in nude mice. *KRAS* (red) and *PIK3CA* (green) probes were prepared as in Materials and Methods.

Figure S3. Knock out of mutant *PIK3CA* affects tumorigenicity in nude mouse xenograft assays. HCT-116 cells with a single copy of wild type *PIK3CA* (HCT-116 PIK3CA WT) or a single copy of mutant *PIK3CA* H1047R (HCT-116 PIK3CA MUT) were inoculated and grown as xenografts in nude mice as described in Materials and Methods. Results are average tumor volumes from ten mice in each group and represent three independent experiments. Error bars represent the SEM. Results between groups are statistically significant at days 11, 15, 18 and 22 with $p<0.001$.

Figure S4. DKI cell lines show phosphorylation of Akt and Erk in physiologic but not supra-physiologic concentrations of EGF.
Western blot illustrating levels of phosphorylated Akt (Ser-473), total Akt, phosphorylated Erk (Thr-202/Tyr-204), total Erk, in DKI cell lines and parental MCF-10A cells in the presence of varying concentrations of EGF. GAPDH is shown as a loading control.

Figure S5. DKI cells have increased phosphorylation of S6 ribosomal protein but not 4E-BP1. Western blot demonstrating levels of phosphorylated S6 ribosomal protein (Ser 240/245), total S6 ribosomal protein, phosphorylated 4E-BP1 (Thr 37/46), and total 4E-BP1 in cell lines grown in the absence of EGF for DKI cells along with MCF-10A and single knock in controls.

Figure S6. DKI cells are sensitive to the MEK inhibitor U0126 but not to rapamycin and show differential effects on phosphorylation of Akt, Erk, p70S6K and p90RSK.

A, B, Cell proliferation assays were performed as described in Materials and Methods, with MCF-10A cells and/or their knock in derivatives using U0126 at a concentration of 1uM, or rapamycin at a concentration of 1nM without EGF. Data are shown as the percentage of cell proliferation relative to vehicle only (DMSO) controls at day 6. Error bars represent the standard error of the mean from triplicate samples. * $p<0.001$, ** = not statistically significant for comparison between groups.
C, Western blot demonstrating levels of phosphorylated p70S6K (Thr389), total p70S6K, phosphorylated p90RSK (Ser380), and total p90RSK in the absence of EGF with U0126 (1uM) or rapamycin (1nM) in DKI cells along with MCF-10A controls. GAPDH is shown as a loading control.
D, Western blot demonstrating phosphorylated Akt (Ser-473), total Akt, phosphorylated Erk (Thr-202/Tyr-204), total Erk, with and without rapamycin (1nM) (top panel), and with and without U0126 (1uM) (bottom panel) in DKI cell lines in the absence of EGF. GAPDH is shown as a loading control.
E, Cell proliferation assays were performed as described in Materials and Methods, with MCF-10A cells and/or their knock in derivatives using rapamycin at a concentration of 1nM with 0.2ng/ml of EGF. Data are shown as the percentage of cell proliferation relative to vehicle only (DMSO) controls at day 6. Error bars represent the standard error of the mean from triplicate samples. * $p<0.001$, for comparison between groups.
F, Western blot demonstrating levels of phosphorylated Akt (Ser-473), total Akt, phosphorylated p70S6K (Thr389), total p70S6K, phosphorylated Erk (Thr-202/Tyr-204), total Erk, phosphorylated p90RSK (Ser380), and total p90RSK grown in the absence of EGF with and without rapamycin (1nM) and U0126 (1uM) in DKI cells along with MCF-10A controls. GAPDH is shown as a loading control.
G, Cell proliferation assays were performed as described in Materials and Methods, with MCF-10A cells and/or their knock in derivatives using U0126 at a concentration of 1uM and rapamycin at a concentration of 1nM without EGF. Data are shown as the percentage of cell proliferation relative to vehicle only (DMSO) controls at day 6. Error bars represent the standard error of the mean from triplicate samples. ** = not statistically significant for comparison between groups.

Figure S7. Transgene expression of mutant *PIK3CA* cDNA with and without RBD mutations in MCF-10A and *KRAS* G12V knock in cell lines.
A, Equal ratios of mutant to wild type *PIK3CA* gene expression is observed two weeks after retroviral infection of mutant *PIK3CA* cDNA as shown by the presence of equivalent heterozygous mutant and wild type alleles using direct sequencing of RT-PCR products. Point mutations for *PIK3CA* E545K and H1047R cDNAs are denoted beneath the wild type nucleotides for both MCF-10A and *KRAS* G12V cell lines.
B, Equal ratios of mutant to wild type *PIK3CA* gene expression is observed two weeks after retroviral infection of mutant *PIK3CA* cDNA with RBD mutations T208D and K227A as shown by the presence of equivalent heterozygous mutant and wild type alleles using direct sequencing of RT-PCR products. Introduced RBD point mutations are denoted beneath the wild type nucleotides for both MCF-10A and *KRAS* G12V cell lines and are representative for both E545K and H1047R cDNA constructs.

Figure S8. Pdk1 activity is increased in DKI cells.
A, Western blot demonstrating levels of phosphorylated Pdk1(Ser241) for both polyclonal and monoclonal antibodies and total Pdk1in DKI cells along with MCF-10A and single knock in controls. GAPDH is shown as a loading control.
B, Western blot demonstrating levels of phosphorylated p90RSK (Ser221) and total p90RSK grown in the absence of EGF without (top) and with (bottom) the Pdk1 inhibitor GSK2334470 (PDKi 5μM) in DKI cells along with MCF-10A and single knock in controls. GAPDH is shown as a loading control.
C, Cell proliferation assays were performed as described in Materials and Methods, with MCF-10A cells and/or their knock in derivatives using the Pdk1 inhibitor GSK2334470 at a concentration of 5uM without EGF. Data are shown as the percentage of cell proliferation relative to vehicle only (DMSO) controls at day 6. Error bars represent the standard error of the mean from triplicate samples. * $p<0.01$, for comparison between groups.

Figure S9. Microsatellite (MIN) colorectal cancers with mutant *KRAS* and mutant *PIK3CA* have single copies of mutant alleles. Colorectal cancer patient samples with known MIN status and mutant *KRAS* and mutant *PIK3CA* status were sequenced to compare relative ratios of mutant to wild type alleles. *KRAS* (red) and *PIK3CA* (green) specific probes were then used for FISH. Note for Patient Sample #4 non-MIN tumor, the ratio of mutant (G12C) to wild type *KRAS* is 1:1, however FISH demonstrates 4 total copies of the *KRAS* gene. Point mutations are denoted beneath the wild-type nucleotides.

Supplementary Tables

Table S1. Knock in cell lines used in this study

Cell line	Description
MCF-10A	Parental MCF-10A cell line
KRAS G12V	MCF-10A with single *KRAS* G12V knock in allele
PIK3CA E545K	MCF-10A with single *PIK3CA* E545K knock in allele
PIK3CA H1047R	MCF-10A with single *PIK3CA* H1047R knock in allele
DKI2	*KRAS* G12V with single *PIK3CA* E545K knock in allele
DKI3	*KRAS* G12V with single *PIK3CA* E545K knock in allele
DKI5	*KRAS* G12V with single *PIK3CA* H1047R knock in allele
DKI6	*KRAS* G12V with single *PIK3CA* H1047R knock in allele
Targeted WT	*KRAS* G12V with single *PIK3CA* E545E knock in allele (control)
RI2	*KRAS* G12V with random integration of *PIK3CA* E545K targeting construct (control)
RI5	*KRAS* G12V with random integration of *PIK3CA* H1047R targeting construct (control)

Table S2. Cell cycle analysis of DKI clones and control cells in the absence of EGF

CELL LINE	G0/G1 (%)	S (%)	G2/M (%)
MCF-10A	94.82	1.86	3.36
KRAS G12V	90.79	4.18	5.57
PIK3CA E545K	79.54	11.49	10.27
PIK3CA H1047R	70.71	13.35	16.88
DKI2	58.21	24.57	18.22
DKI3	58.79	22.27	19.93
DKI5	69.06	17.29	14.52
DKI6	71.20	17.00	13.10

Table S3. Colony formation assay

Cell line	**(% = colonies/plated cells)**
MCF-10A	**0**
***KRAS* G12V**	**0**
***PIK3CA* E545K**	**0**
***PIK3CA* H1047R**	**0**
DKI2	**11**
DKI3	**13**
DKI5	**7**
DKI6	**9**
Targeted WT	**0**
RI2	**0**
RI5	**0**
LXSN *KRAS* G12V	**22**

Table S4. Gene targeting of p110α RBD missense mutations

CELL LINE	*KRAS* mutation status	*PIK3CA* mutation status	RBD/*PIK3CA* mutation in cis post gene targeting (same allele)	RBD/*PIK3CA* mutation in trans post gene targeting (opposite alleles)
HCT-116	G13D	H1047R	0	8*
DLD1	G13D	E545K	0	7*

*p<0.0001

Table S5. Gene mutations in colon cancer samples in relation to *KRAS* mutational status

Mutation	*KRAS* wild type (n=18)	*KRAS* mutant (Heterozygous) (n=17)	*KRAS* mutant* (Homozygous) (n=7)
PIK3CA	4 (22%)	8 (47%)	0 (0%)
BRAF	7 (39%)	0 (0%)	0 (0%)
B-catenin	1 (6%)	2 (12%)	0 (0%)
APC	11 (61%)	12 (71%)	2 (29%)
TP53	9 (50%)	6 (35%)	6 (86%)
SMAD4	3 (17%)	2 (12%)	2 (29%)
CDKN2A	1 (6%)	2 (12%)	0 (0%)
PTEN	1 (6%)	0 (0%)	0 (0%)
RB	0 (0%)	1 (6%)	0 (0%)

*p<0.05 comparing mutations in other oncogenes between *KRAS* mutant homozygous vs. *KRAS* wild type and *KRAS* mutant heterozygous samples

Table S6. Primers used in this study

Screen	Primer	Sequence
PIK3CA E545K Pre-Cre Across 5' Homology Arm	FWD	ATCTCTTTCCTGGACTACTGG
	REV	GCAGACAGCGAATTAATTCC
PIK3CA E545K Pre-Cre Across 3' Homology Arm	FWD	TTAAGGTACCACTGTGCATATG
	REV	TAAAATTCAAAAGACATCAGTG
PIK3CA E545K Post-Cre Across 5' Homology Arm and Lox P	FWD	ATCTCTTTCCTGGACTACTGG
	REV	CATATGCACAGTGGTACCTTAA
PIK3CA E545K allelic ratio gDNA	FWD	TGAAAATAAAGTCTTGCAATG
	REV	TTAGGCACAGTTATAATGGC
	Seq	AGTAACAGACTAGCTAGAGA
PIK3CA E545K allelic ratio cDNA	FWD	CGACTTTGCCTTTCCATTTG

	REV	GGGTAATTACAGTCCAGAAG
	Seq	CAGGATTTAGCTATTCCCAC
PIK3CA H1047R Pre-Cre Across 5' Homology Arm	FWD	TTTTGGTCATACACTTTGAGG
	REV	GCAGACAGCGAATTAATTCC
PIK3CA H1047R Pre-Cre Across 3' Homology Arm	FWD	TTAAGGTACCACTGTGCATATG
	REV	TAAATGGGATAGTGCCTGAG
PIK3CA H1047R Post-Cre Across 5' Homology Arm and Lox P	FWD	TTTTGGTCATACACTTTGAGG
	REV	CATATGCACAGTGGTACCTTAA
PIK3CA H1047R allelic ratio gDNA	FWD	AAGTCAGTCAACCATAATCACC
	REV	ATTGCGCATTATTCTAAAGC
	Seq	GAAAGCCTCTCTAATTTTGT
PIK3CA H1047R allelic ratio cDNA	FWD	TAACATCATGGTGAAAGACG
	REV	TCAGTTCAATGCATGCTGT
	Seq	TGTTACAAGGCTTATCTAGC
KRAS G12V allelic ratio gDNA	FWD	GTTCTTCTTTGCCTCAGTGTT
	REV	CAGATAAAGGTTTCTCTGACC
	Seq	CGATGGAGGAGTTTGTAAATG
KRAS gDNA quantitative real time PCR	FWD	TCATTACGATACACGTCTGC
	REV	GTTGGATCATATTCGTCCAC
PIK3CA gDNA quantitative real time PCR	FWD	CAGCCCGCTCAGATATAAAC
	REV	CAGAGGATAGCAACATACTTC
GAPDH gDNA quantitative real time PCR	FWD	ACATCATCCCTGCCTCTAC
	REV	TCAAAGGTGGAGGAGTGG